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INDIVIDUALIZED CHEMICAL SYSTEMS MEDICINE OF ACUTE AND CHRONIC MYELOID LEUKEMIA

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ACADEMIC DISSERTATION

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‘*I have no special talents. I am only passionately curious*’
- Albert Einstein

To my family

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ABBREVIATIONS

α-KG	Alpha ketoglutarate
2-HG	Two hydroxyglutarate
AA	Activity Area
ABL	Abelson murine leukemia viral oncogene homolog
ALL	Acute lymphoblastic leukemia
A-loop	Activation loop
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
ATRA	Retinoic acid
AUC	Area under the curve
BAAL	Brain and acute leukemia gene
BCR	Breakpoint cluster region
CBF	Core binding factor
CCLE	Cancer Cell Line Encyclopedia
CEBPα	CCAAT/enhancer binding protein alpha
CGP	Cancer Genome Project
CML	Chronic myeloid leukemia
CNS	Central nervous system
COSMIC	Catalogue of somatic mutations in cancer
CR	Complete remission
CSF1R	Colony-stimulating factor 1 receptor
DMSO	Dimethyl sulfoxide
DNMT3A	DNA methyltransferase 3A
DSRT	Drug sensitivity and resistance testing
DSS	Drug sensitivity score
ELN	European Leukemia Net
EMA	European Medicinal Agency
FAB	French-American-British
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FLT3	Fms-like tyrosine kinase 3
GTP	Guanine triphosphate
HDAC	Histone deacetylase
HSCT	Hematopoietic stem cell transplant
IC_{50}	Half-maximal inhibitory concentration
ISM	Individualized systems medicine
ITD	Internal tandem duplication
JAK	Janus-activated kinase
LOH	Loss of heterozygosity
LT-HSC	Long term hematopoietic stem cell
M-bcr	Major breakpoint cluster region
MCM	Mononuclear cell medium
MDS	Myelodysplastic syndrome
MK	Monosomal karyotype
MLL	Mixed lineage leukemia gene
NCI-60	National Cancer Institute 60 platform
NK	Normal karyotype
NPM1	Nucleophosmin 1 gene

PDGFR	Platelet derived growth factor receptor
Ph+	Philadelphia chromosome positive
PTD	Partial tandem duplication
RARα	Retinoic acid receptor alpha
RTK	Receptor tyrosine kinase
sDSS	Selective drug sensitivity score
SH2	Src-homology-2
WBC	White blood cell
WHO	World Health Organization
WT1	Wilms tumor 1 gene

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. **Pemovska, T**[*]., Kontro, M[*]., Yadav, B., Edgren, H., Eldfors, S., Szwajda, A., Almusa, H., Bespalov, M.M., Ellonen, P., Elonen, E., Gjertsen, B.T., Karjalainen, R., Kulesskiy, E., Lagström, S., Lehto, A., Lepistö, M., Lundán, T., Majumder, M.M., Marti, J.M., Mattila, P., Murumägi, A., Mustjoki, S., Palva, A., Parsons, A., Pirttinen, T., Rämet, M.E., Suvela, M., Turunen, L., Västrik, I., Wolf, M., Knowles, J., Aittokallio, T., Heckman, C.A., Porkka, K., Kallioniemi, O., Wennerberg, K.; Individualized Systems Medicine strategy to tailor treatments for patients with chemorefractory acute myeloid leukemia. 2013, *Cancer Discov*., 3:1416-1429.

II. **Pemovska, T**., Johnson, E., Kontro, M., Repasky, G.A., Chen, J., Wells, P., Cronin, C.N., McTugue, M., Kallioniemi, O., Porkka, K., Murray, B.W., Wennerberg, K.; Axitinib effectively inhibits BCR-ABL1(T315I) with a distinct binding conformation. 2015, *Nature*., 519:102-105.

Supplementary article (SA) I: Yadav, B., **Pemovska, T**., Szwajda, A., Kulesskiy, E., Kontro, M., Karjalainen, R., Majumder, M.M., Malani, D., Murumägi. A., Knowles, J., Porkka, K., Heckman, C., Kallioniemi, O., Wennerberg, K., Aittokallio, T.; Quantitative scoring of differential drug sensitivity for individually optimized anticancer therapies. 2014, *Sci Rep*., 4:5193.

*Equal contribution

ABSTRACT

Adult acute myeloid leukemia (AML) effectively illustrates the challenges of contemporary cancer drug discovery and development, as molecularly targeted therapies have not yet been translated in clinical practice. In fact the standard therapy (cytarabine and an anthracycline) for AML has not been changed in over 40 years. As a consequence, outcome remains poor with overall survival of 30-40%. The genetic alterations that are associated with AML have been mapped out, but the underlying disease mechanism is poorly defined due to large inter-patient heterogeneity. In contrast, chronic myeloid leukemia (CML) is strictly driven by BCR-ABL1 and drugs targeting the ABL1 kinase activity have paved the way for oncoprotein targeting drugs in the treatment of cancer. In CML the main clinical challenge is instead the emergence of resistance to ABL1-directed therapy. This resistance typically occurs through point mutations in the kinase domain of ABL1 such as the clinically challenging T315I mutation. Hence, in both leukemia types there is an unmet need for novel therapeutic strategies.

This study focused on development and implementation of an Individualized Systems Medicine (ISM) platform to identify novel therapeutic strategies for leukemia patients. The ISM strategy incorporated functional *ex vivo* drug sensitivity and resistance testing (DSRT), deep molecular profiling and clinical information to facilitate identification of personalized therapy approaches. A large number of approved and investigational anti-cancer compounds were tested and individualized selective responses were quantified with drug sensitivity scores (DSS). RNA and exome sequencing data was used to identify genetic alterations that enabled associating drug sensitivities with genetic alterations and biomarkers.

The DSRT approach enabled functional taxonomy of AML patient samples based on drug responses, provided insights into disease biology, and identified effective drugs and drug combinations for individual patients and thus facilitated drug repurposing. In addition, integration of DSRT and molecular data identified phenotype to genotype links that has a potential for rapid translation of results. Clinical implementation of ISM data was plausible in approximately 80% of relapsed and refractory AML patient cases, and meaningful and evaluable responses were achieved in approximately 40% of cases. Notably, emergence of *in vivo* resistance to targeted therapy was mirrored in the DSRT profile of the relapsed samples, highlighting a solid correlation between *ex vivo* and *in vivo* drug responses. Finally, this study identified a number of kinase inhibitors that can be repositioned for specific subtypes of AML and CML, such as dasatinib in combination with a FLT3 inhibitor for AML patients with *FLT3*-ITD mutations and axitinib for patients with BCR-ABL1(T315I)-driven leukemia.

The results of this thesis demonstrate how unbiased drug sensitivity profiling of patient-derived cancer cells is a powerful way to discern unforeseen drug-disease and drug-target links with clinical implications and provides a workable concept that can be implemented in routine clinical care of cancer patients in the future.

INTRODUCTION

The molecular characterization of various cancer types has dominated the cancer research field in recent years. However, this information has not led to significant advances in cancer therapy and clinical outcome of patients as judged by increase in cancer incidence, drug resistance, and underlying genetic complexity and heterogeneity[1-4]. Our functional understanding of the fundamental disease mechanisms is very limited and it is imperative to develop ways to systematically study cancer patient cells in order to effectively link functional profiles to cancer genotypes. Moreover, majority of cancers do not harbor druggable genetic aberrations that can be directly associated with existing drugs. For instance, mutations in the *RAS* genes occur in over 30% of cancers and yet its therapeutic targeting remains a significant challenge[5]. Thus, genotype to phenotype translation is not a simple task as even when drugs exist for a particular target, the cancer cells are predominantly not sensitive to direct inhibition of the oncoprotein due to cell plasticity, tumor heterogeneity, and compensatory signals[6]. Although comprehensive efforts to identify cancer-relevant genes are now routine, the assessment for their potential therapeutic targeting is largely an impromptu exercise not fully utilizing the vast array of data available[7].

Since there is a significant inter and intra-patient heterogeneity in cancer, individualized and combinatorial treatments will be necessary to attain meaningful responses. Customizable treatment strategies might be difficult to forecast only based on the genomic attributes of the cancer cells. Thus, it is evident that there is a need to extend the structural understanding of cancer genomes and supplement it with functional profiling of cancer cells[6]. There is an increasing number of molecularly targeted drugs available pre-clinically and clinically and this provides an unprecedented opportunity to comprehensively profile individual cancer patient samples in terms of molecular drivers, molecular mechanisms of disease as wells as personalized therapy options. Phenotypic drug sensitivity can aid in reading the cancer genome and provide a suitable alternative to the classical genotype to phenotype dogma where functional profiles can link to genetic alteration patterns. This strategy has potential for immediate translation of results especially when combined with clinical patient information.

To gain a functional understanding of cancer patient cells, this study aimed to develop and implement a novel *ex vivo* high throughput drug sensitivity and resistance testing (DSRT) screening platform that covers the entire approved cancer pharmacopeia and emerging investigational anti-cancer drugs. More specifically the goal was to identify: underlying signaling pathways and processes driving cancer progression; individualized therapy options; mechanisms of drug sensitivity and resistance; and cancer drugs that can be repurposed for other cancer indications. This study focused on patients with hematological malignancies such as acute and chronic myeloid leukemia, for which there is a need of novel treatment strategies as well as a deeper understanding of drug resistance and disease progression mechanisms.

REVIEW OF THE LITERATURE

1. Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a hematological malignancy affecting blood cells committed to the myeloid linage of development. The disease is defined by the cancerous conversion of bone marrow-derived progenitor cells, which are characterized with a reduced rate of self-apoptosis and aberrant differentiation. In AML, rapid growth of abnormal, immature white blood cells (WBC; known as blasts) overpopulates the bone marrow and interferes with normal hematopoiesis[8,9] (Figure 1). According to the World Health Organization (WHO) criteria[10] at least 20% blasts of the myeloid lineage have to be detected in the bone marrow of a patient to warrant an AML diagnosis. AML is a heterogeneous clonal disease that is often accompanied with accumulation of somatically acquired gene mutations in hematopoietic progenitor cells, which lead to changes in cell renewal, proliferation and differentiation translating to differences in response to therapy and overall survival[11,12]. Disease progression in AML is very rapid and can be deadly within weeks or months if not treated. Patients most commonly succumb to the disease due to bone marrow failure[13].

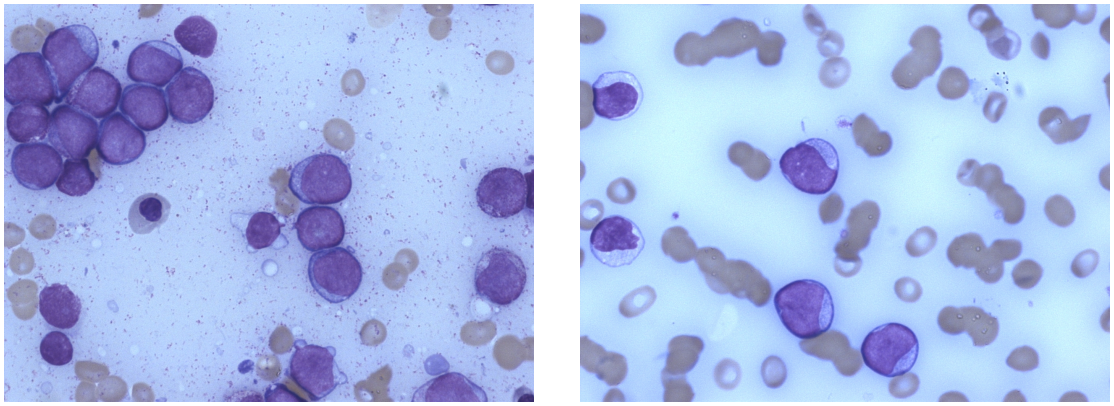


***Figure 1**. Giemsa staining of acute myeloid leukemia blasts in bone marrow (left) and peripheral blood (right). Courtesy of Satu Mustjoki.*

AML is the most common type of acute leukemia in adults (accounting for 25% of all leukemias) and its incidence increases with age with the mean age of diagnosis of 67-70[14]. According to most recent statistics, AML is diagnosed in approximately 21,000 individuals per year in the United States and results in approximately 10,500 deaths. Moreover, AML is more prevalent in males than in females with male-to-female ratio of 1.5:1[15].

1.1 Etiology, signs and symptoms of AML

Several risk factors have been described for AML onset. However, only a small fraction of AML cases can be attributed to known risk factors such as age, previous hematological disorders, genetic conditions, exposure to radiation, harmful substances (e.g. benzene) or chemotherapy. Majority of AML patients present with weight-loss, unexplainable fatigue and fever. Most of the symptoms can be explained by bone marrow deterioration such as

anemia, neutropenia, and thrombocytopenia. AML diagnosis can be obtained following blood cell count evaluation as well as profiling of the bone marrow aspirates/biopsies by microscopy, flow cytometry, immunohistochemistry, and cytogenetics[14,16].

1.2 Therapy and treatment outcome in AML

The standard therapy for AML has not been changed in over 40 years and primarily consists of cytarabine and an anthracycline (e.g. idarubicin, daunorubicin or doxorubicin)[9,17,18]. The main goal of AML treatment is to achieve and preserve complete remission (CR), which is defined as bone marrow with less than 5% blasts. Treatment of AML is carried out in two phases, namely remission induction therapy and post-remission therapy. During induction therapy, a reduction of the number of malignant cells in the bone marrow is desired so that normal blood cell production can be resumed. Using standard therapy, approximately 65-85% of younger patients and less than 50% of older patients achieve remission[19,20]. However, long-term survival following remission induction therapy is only 30% with treatment related mortality of 5-10%. Post-induction therapy most commonly consists of standard dose cytarabine or hematopoietic stem cell transplantation (HSCT) for high-risk patients if a donor match is found. Two types of post-remission therapy have been characterized: consolidation therapy and maintenance therapy. Consolidation therapy is necessary to achieve prolonged remission, since patients that do not receive it most commonly relapse in 6-9 months[21,22]. Maintenance therapy, normally perceived as less myelosuppressive, in comparison to induction or consolidation therapy is given to patients that have already achieved CR. Its main aim is to prevent a relapse and to further reduce the number of the remaining leukemic cells.

Even though there has been considerable progress in improving the care and treatment options of newly diagnosed AML patients, still 20-40% of patients never achieve CR with standard induction therapy and in addition 50-70% of patients that do achieve CR relapse within 3 years[23]. Prognosis of patients that relapse following a CR is poor. The length of the first remission is a relatively good predictor of the likelihood of a second CR and survival[24]. Moreover, possible treatment options for AML patients are heavily dependent on the age of the patient, as older patients have significantly lower survival and remission rates, experience more treatment related toxicity, have shorter disease free survival and shorter overall survival times[12,25,26].

Efforts have been made to identify alternative treatment strategies that can improve the remission rate and quality thereof, but have been largely unsuccessful to date. Although a large percentage of AML patients under the age of 60 achieve CR following standard anthracycline and cytarabine-based therapy, long-term survival is fairly poor with only 40% of patients being alive at 5 years[27,28]. The long-term prognosis for high-risk AML patients (defined as either older than 65, having had previous myelodysplastic syndrome (MDS) or having secondary AML due to environmental exposures or previous treatment with chemotherapy) is even poorer with CR achieved in less than 40% of the cases and survival rates of less than 10%[13,29,30]. Approximately 10-14% of

patients will fail induction chemotherapy due to either treatment complications or refractory disease. A large proportion of patients achieve remission following the first induction therapy. The degree of blast cell reduction during initial course of chemotherapy has significant prognostic ramifications for disease relapse following remission. Hence, lower blast reduction confers higher relapse risk[31,32].

2. Classification of AML

The French-American-British (FAB) Cooperative Group established the initial classification system for AML[33]. This system recognizes eight different AML subtypes (M0-M7), largely based on morphology and immunohistochemistry of lineage markers (Table 1)[34]. The FAB classification required 30% blasts to be present in the bone marrow for an AML diagnosis. However in 1999 a new system for disease stratification was introduced by the WHO, which reduced the blast minimum to 20%[10,35].

***Table 1**. Classification of AML based on French-American-British (FAB) criteria*

Subtype	Morphological features	Prevalence %
AML-M0	Undifferentiated acute myeloblastic leukemia	5
AML-M1	Acute myeloblastic leukemia with minimal maturation	15
AML-M2	Acute myeloblastic leukemia with maturation	25
AML-M3	Acute promyelocytic leukemia	10
AML-M4	Acute myelomonocytic leukemia	20
AML-M4 eos	Acute myelomonocytic leukemia with eosinophilia	5
AML-M5	Acute monocytic leukemia	10
AML-M6	Acute erythroid leukemia	5
AML-M7	Acute megakaryoblastic leukemia	5

Modified from the review by Chandra Kumar, Genes Cancer, 2011[13]

The WHO system includes morphology as well as novel prognostic markers such as cytogenetics, molecular genetics, immunologic markers, and clinical features to divide patients into subgroups that have prognostic and therapeutic implications. Four broad AML subtypes are defined in the WHO classification: 1) AML with recurrent genetic abnormalities; 2) AML with multilineage dysplasia; 3) therapy related AML and MDS; and 4) those that do not fall in any of the above mentioned groups (largely encompassing the FAB subtypes; Table 2)[13,35]. This classification scheme created over 17 different subclasses of AML, facilitating clinicians to identify patient's subgroups that can benefit from specific therapies.

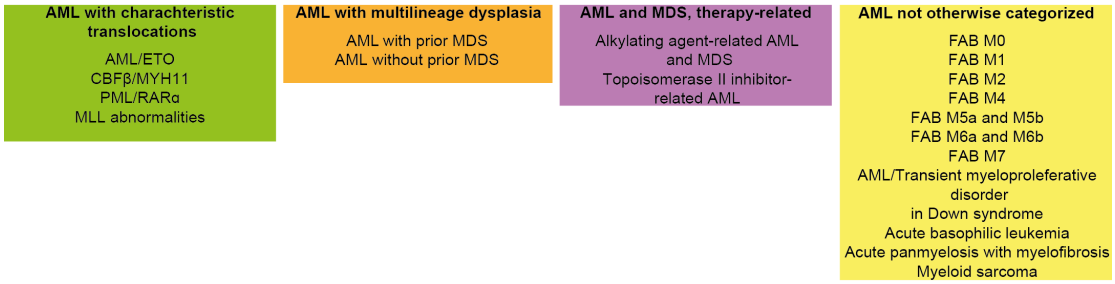


***Figure 2**. The WHO stratification of AML. AML/ETO (RUNX1/MTG8); CBF-core binding factor; RARα-retinoic acid receptor alpha; MLL- Myeloid/lymphoid or mixed-lineage leukemia; MDS-myelodysplastic syndrome.*

2.1 Cytogenetics in AML and its prognostic relevance

As briefly touched upon previously, AML is a highly heterogeneous disease in terms of chromosomal abnormalities, gene mutations, and changes in gene expression. Close to 60% of all newly diagnosed patients have cytogenetic abnormalities such as nonrandom chromosomal translocations leading to gene rearrangements[36]. Currently the strongest independent predictor of AML patient outcome can be derived from cytogenetic and mutational analysis. Gene expression changes also represent important prognostic factors in terms of remission rate, relapse and overall survival[13,36]. Based on cytogenetics, AML patients can be divided in three large prognostic risk groups: favorable, intermediate and adverse[27,37] (Table 2).

2.2 Cytogenetic abnormalities with favorable prognosis

Patients with favorable prognosis are ones with core-binding factor (CBF) leukemias, inv(16)/t(16;16) (CBFB-MYH11) or t(8;21) (RUNX1-RUNXIT1), accounting for 6-10% of cases, respectively[18]. These genetic aberrations lead to disruption of genes coding for distinct subunits of CBF[38]. Patients with these genetic changes typically do not require HSCT, as chemotherapy alone is sufficient for disease control. Roughly 30% of patients with CBF leukemias harbor activating mutations in *KIT* that confer adverse prognosis, lower remission durations and overall survival in comparison to CBF patients with wild-type *KIT*[39,40]. *KIT* mutations lead to constitutive activation of the receptor without the need of ligand. In addition to *KIT* mutations, Fms-like tyrosine kinase 3 (*FLT3*) activating internal tandem duplications (ITDs) have prognostically negative impact in CBF patients[41]. Another subgroup of favorable risk AML are patients with acute promyelocytic leukemia (APL) harboring the PML-RARα t(15;17) rearrangement, which disturbs the natural interaction of retinoic acid with RARα preventing RARα to be transformed to a transcription activator[42]. Patients have sustained long-term remissions and exceptional overall survival when treated with high-dose all-trans retinoic acid (ATRA; tretinoin), which specifically targets PML-RARα thereby overcoming the fusion-induced differentiation block. Patients that relapse or are refractory to ATRA can be salvaged with arsenic trioxide, which induces differentiation of the leukemic cells by degrading the abnormal fusion gene encoded protein[43,44]. Hence, it is vital to rapidly identify patients with APL and ensure that they receive appropriate treatment.

2.3 Normal karyotype AML

The largest subgroup of AML patients (≈45%) exhibits a normal karyotype (NK) and falls into the intermediate risk group[45]. This disease group is very heterogeneous, mostly as a result of vast variability in gene mutations and gene expression[46,47]. Over a decade a ago, a 'two hit model' of leukemia pathogenesis was proposed[48] with two broadly defined categories of gene mutations: class I that activate signaling pathways and in term stimulate hematopoietic progenitor cell proliferation and/or survival (e.g. mutations in *KIT*, *FLT3*, and *RAS*)[39,49-51] and class II that influence transcription factors or components of the cell cycle machinery thereby affecting differentiation (e.g. CBF leukemia, APL, mutations in myeloid/lymphoid or mixed lineage leukemia (*MLL)* gene, brain and acute leukemia (*BAAL)* gene, Wilms tumor (*WT1)* gene, enhancer-binding protein α (*CEBPα*), nucleophosmin 1 (*NPM1)*, and DNA methyltransferase (*DNMT3A)* gene)[52-55]. Often, cooperation between different mutations is seen in the pathogenesis of AML and this process is not random, as particular class I mutations preferentially cooperate with specific class II mutations[56-58]. Thus, enhanced molecular understanding of AML provides the basis for more comprehensive subclassification of the disease and sets postulates of prognosis estimation and therapy stratification. Currently, only mutations in *NPM1*, *CEBRα* and *FLT3* are routinely tested in the clinic and have implications for patient care.

2.4 AML with *NPM1* mutations

The most common mutation in NK patients is in the *NPM1* gene, occurring in 50% of cases. The overall prevalence of *NPM1* mutations in adult AML is 30%. The *NPM1* gene encodes for a nucleolar phosphoprotein (nucleophosmin) that travels between the nucleus and cytoplasm and mutation in the gene influences the intracellular localization of NPM1. The mutation arises as a result of four base pair insertion at position 960 (exon 12 of the gene) leading to an amplified export of the protein from the nucleus to the cytoplasm due to disruption of a C-terminal nuclear localization signal and creation of a new one[16,59,60]. Patients with mutated *NPM1* present with high WBC count and growing number of circulating blasts with monocytic morphology. *NPM1* mutations have been detected in leukemia-initiating cells and typically are stable over the disease course, indicating that these mutations occur early in AML development. However, the exact mechanism of NPM1-influenced leukemogenesis has not been elucidated to date. Overall, patients carrying the *NPM1* mutation have favorable outcome with comparable prognosis as CBF AML[61,62]. In contrast, cooperation with *FLT3*-ITD mutations (but not other cytogenetic abnormalities) confers poorer outlooks for patients[62,63]. Currently no targeted therapies against *NPM1* mutated AML have been developed and usually patients are treated with standard induction therapy and several cycles of high-dose cytarabine as consolidation therapy[64].

2.5 AML with *FLT3* mutations

Twenty to thirty percent of AML patients harbor activating *FLT3*-ITDs in the juxtamembrane domain. Patients present with increased WBC count and typically have NK and therefore fall in the intermediate risk subgroup. In addition, activating tyrosine kinase domain mutations of *FLT3* (*FLT3*-TKD) localized in the activation loop of the kinase have been detected. *FLT3*-TKD are less frequent (5-10%) than *FLT3*-ITD and their prognostic relevance is less clear, but appears to depend on cooperating mutations and genetic background[65,66]. *FLT3* encodes for the receptor tyrosine kinase (RTK) FLT3 belonging to the class 3 family of RTKs, which is targetable with small molecule kinase inhibitors[67]. Upon FLT3 ligand binding, FLT3 gets activated causing the instigation of downstream effector signaling pathways (e.g. STAT5, RAS, PI3K) thereby promoting the survival and proliferation of leukemic cells. Both alterations cause autophosphorylation of the FLT3 receptor that in turn leads to increased cell proliferation, inhibition of apoptosis and activation of signaling pathways independent of a ligand[68,69]. The prognosis of NK-AML with *FLT3*-ITD is significantly worse than NK-AML patients without the mutation when treated with standard therapy[70]. Evidence exists that the outcome of FLT3-driven disease is linked with the ratio of mutant vs. wild-type allele where high allelic burden of the mutant envisages poorer survival[37]. Targeted therapies for FLT3 have been developed and are currently being investigated in clinical trials with quizartinib being the most potent and furthest in clinical development (currently in phase 3 trials for refractory AML patients with *FLT3*-ITD mutations; NCT02039726). In addition, HSCT could be a viable treatment option to consider in patients with *FLT3*-ITD-positive AML[45].

2.6 AML with *CEBPα*

CEBRα falls in the family of basic region leucine zipper transcription factors and influences granulocyte differentiation of common myeloid progenitors[71]. The frequency of *CEBPα* mutations in AML is approximately 5-15%, with 50% of mutations clustering in the N-terminus causing either a truncated nonfunctional protein or increased expression of the dominant-negative isoform of $p30^{CEBP\alpha}$. In contrast, mutations in the C-terminus result in an abnormal protein lacking DNA binding and/or homodimerization capabilities[46,72]. In 75% of cases, N- and C-terminus mutations are biallelic with most being compound heterozygous (N-terminal on one allele and C-terminus on the other). *CEBPα* mutations are usually detected in NK-AML and in patients with 9q deletion[9]. Patients harboring *CEBPα* mutations generally have good clinical outcome analogous to AML patients with *NPM1* mutations without *FLT3*-ITD[70] (Figure 3). Interestingly, only biallelic *CEBPα* mutations are associated with favorable prognosis, whereas the survival of patients with monoallelic *CEBPα* mutations is comparable to AML patients with wild-type *CEBPα*[72]. This variable disease outlook is likely due to distinct gene expression (e.g. downregulation of *HOX* genes) and methylation pattern in double mutant cases, data for which comes from disease modeling in mice[73,74]. No targeted therapy is available for *CEBPα* mutant AML and the therapy recommendation is in line with that for *NPM1*-driven AML without

FLT3-ITD. There are not clear indications that patients with *CEBPα* mutations benefit from HSCT in first CR.

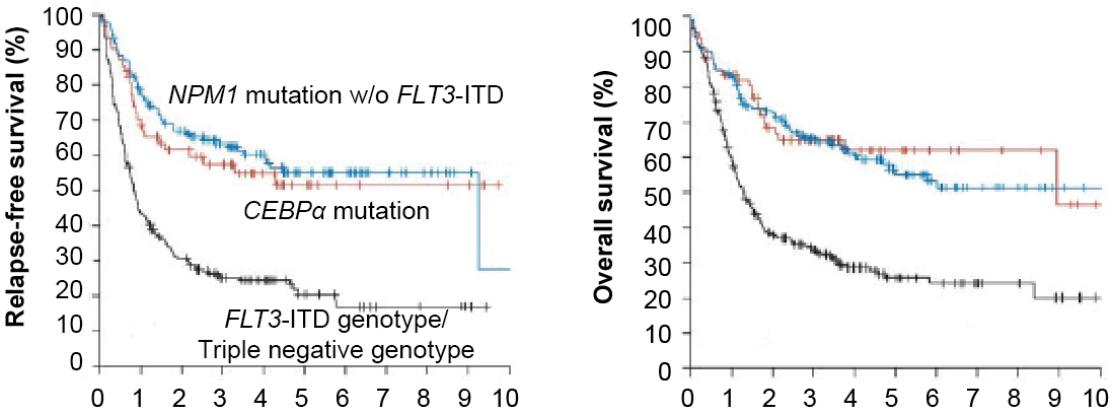


***Figure 3**. Kaplan–Meier curves of relapse-free survival and overall survival of AML patients with: NPM1 mutations without FLT3-ITD (blue curve), biallelic CEBPα mutations (red), and with either FLT3-ITD or triple negative genotype (wild type NPM1 and CEPBα, and no FLT3-ITD; black). Adapted from Schlenk et al, N Engl J Med, 2008[62].*

2.7 Other molecular markers commonly identified in NK-AML

The majority of patients (≈85%) with NK AML have a mutation or other genetic abnormality[12], but their prognostic relevance is uncertain. In addition to *NPM1*, *CEBPα* and *FLT3*-ITD mutations, several other molecular markers are gaining prognostic and therapeutic importance such as *DNMT3a, IDH1/2, TET2* and *WT1*.

DNMT3a mutations

DNMT3a mutations were identified by massive parallel sequencing of the genome of a NK AML patient[55]. DNMT3a is a DNA methytransferase that is responsible for adding methyl groups to cytosine residues of CpG nucleotides[75]. The frequency of *DNMT3a* mutations in de novo AML patients is 22-26%[55,76]. There has been an array of missense, nonsense, frame-shift, and splice-site mutations detected along the gene and they most frequently cluster at position R882[55]. The functional implication of *DNMT3a* mutations is still unclear but some reports exist that they result in either reduced DNA methylation activity (R882 mutations)[77] or untimely truncation of the encoded protein (non-R882 mutations)[75,78]. There have been conflicting reports regarding the effect of *DNMT3a* mutations on DNA methylation and gene expression in AML. Ley and colleagues have found no tangible changes in methylation and gene expression[55], whereas others have detected increased expression of *HOX* family genes[77] or strong association between DNA methylation and *DNMT3a* mutation status[79]. Hence, it is largely unknown how this mutation impacts AML pathogenesis. However, recent large scale sequencing initiatives have determined that approximately 44% of AML patient samples harbor mutations in DNA methylation associated genes[76], suggesting that abnormal DNA methylation is characteristic of AML and likely plays a role in the underlying mechanism of the disease[80,81].

DNMT3a mutations confer poor outcome and do not occur in patients with favorable cytogenetics. Thus, mutations in *DNTM3a* primarily are detected in intermediate-risk patients (NK patients) with overall somatic mutation rate of 12-35[78]. *DNMT3a* mutations commonly cooperate with *NPM1*, *FLT3*-ITD and *IDH1* mutations, but are mutually exclusive with transcription factor fusions (e.g. PML-RARα), suggesting similar functions in initiation of AML pathogenesis[76]. Moreover, a study by Patel et al observed mutual exclusivity between *DNMT3a* mutations and MLL fusions[82]. In recent years, there has been increasing interest in determining the value of hypomethylating agents in patients with aberrant methylation. Decitabine and azacitidine are DNA-methyltransferase inhibitors, which are clinically available for treatment of high-risk MDS. Decitabine exhibits its action by incorporating into DNA and blocking DNA synthesis, proliferation and stimulating apoptosis and myeloid differentiation[83,84]. Azacitidine, on the other hand, can incorporate into both DNA and RNA thus inhibiting DNA, RNA and protein synthesis and can also influence myeloid differentiation[85]. Their DNA incorporation ability occurs at sub-toxic doses by forming irreversible complexes with DNMT1 thereby sequestering and inhibiting the enzyme. In contrast, blocking of DNA and RNA synthesis is achieved at higher doses. Hence, these two drugs dot not inhibit DNMT3a directly[86,87]. These agents have been evaluated in clinical trials for AML patients with mixed results[88-91]. Decitabine and azacitidine have a favorable safety profile and potential clinical benefit, deserving further evaluation either as monotherapy or in combination with other drugs especially in older patient population unfit for standard induction chemotherapy.

IDH1/2 mutations

The importance of *IDH1/2* mutations was first recognized in glioma and secondary glioblastoma, where the incidence is 70%[92]. More recently they have also been identified in AML with frequency of 6-16% and 8-19% for *IDH1* and *IDH2*, respectively[76,93-95]. Similarly as with *DNMT3a*, these mutations are increasingly found in patients with normal cytogenetics. Interestingly, *IDH* genes encode for metabolic enzymes playing a role in citrate metabolism, a crucial part of the Krebs cycle. Their normal function is to facilitate the oxidative decarboxylation of isocitrate giving rise to α-ketoglutarate (α-KG). IDH1/2 mutations are typically heterozygous, implying that they lead to enzymatic gain of function[78] and indeed, the encoded oncoproteins attain a different enzymatic activity by which α-KG is reduced to 2-hydroxyglutarate (2HG), causing a rise of 2HG levels that inhibit α-KG-dependent reactions vital for normal DNA methylation[96,97]. IDH1/2 mutant protein expression negatively influences myeloid differentiation and leads to increased expression of stem cell markers, alluding to a role in block of differentiation of AML cells. Moreover, a signature of significant gain in methylation has been associated with *IDH1/2* mutant AML[76,98].

IDH1/2 mutations often cooperate with *NPM1* mutations, but are mutually exclusive with *TET2* mutations. Conflicting reports, regarding the prognosis of AML patients with *IDH1/2* mutation, have been published with some finding associations with worse prognosis, others with good or no association at all[78,82]. Patel et al found that co-occurring *NPM1* and *IDH* mutations without

FLT3-ITD had a more favorable outcome than *NPM1* mutant *FLT3*-ITD negative patients[82]. Hence, the bearing of *IDH1/2* mutations on clinical outcome may be contingent on specific patient populations. Several IDH inhibitors have been developed and are currently in different stages of preclinical and clinical evaluation. Preclinical studies have shown that IDH inhibitors act by either reducing the hypermethylation states of histones and DNA, lowering the 2HG levels, preventing IDH1 to produce 2HG, or promoting differentiation[99-101]. Of note, AG-881, AG-120 and AG-221 are currently undergoing phase I studies in advanced hematologic malignancies with IDH1 or IDH2 mutations (NCT02492737), IDH1 mutations only (NCT02074839), and IDH2 mutations only (NCT01915498), respectively.

TET2 mutations

TET2 mutations were first described in 2009 and are detected in 20% of MDS and secondary AML patients[102] as well as 10-20% of AML patients. TET2 is a metabolic enzyme that catalyzes the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, which plays a role in DNA demethylation control[103,104]. Different types of *TET2* mutations have been detected such as deletions, nonsense mutations and missense mutations, but their functional significance it unclear though they have been predicted to be inactivating[102]. In most patients heterozygous mutations have been detected, suggesting that TET2 haploinsufficiency is sufficient to maintain the leukemia. *TET2* mutant AML exhibits a gene hypermethylation phenotype alike *IDH1/2* mutated AML[98]. *TET2* mutations frequently co-occur with *NPM1* and *DNMT3a* mutations, but are mutually exclusive with MLL-PTD and *IDH1/2* mutations. A recent study has shown that *TET2* mutations are not early leukemia initiating events, but rather occur later than *NPM1* and *DNMT3a*[105].

WT1 mutations

Five to thirteen percent of AML patients have mutations in the *WT1* gene with enrichment in NK-AML[76,82,106]. *WT1* encodes for a zinc-finger transcription factor involved in control of apoptosis, proliferation and differentiation of hematopoietic cells. The role of *WT1* is paradoxical in that it was originally thought to be a tumor suppressor gene, but oncogenic roles have also been recognized[107]. The majority of large-scale studies indicate a poor prognosis association of *WT1* mutations with reduced relapse-free survival and overall survival in comparison to *WT1* wild-type patients, but this has not been corroborated in all studies likely due to differences in patient populations and presence of additional mutations[108-110]. Moreover, *WT1* overexpression is detected in large proportion of AML patients. A recent study found that *WT1* mutations are predominant in females, younger patients, good outcome subgroups of patients with *PML-RARα* or biallelic *CEBPα,* and in patients with *FLT3*-ITD[106]. In contrast, mutual exclusivity was observed with *ASXL1*, *IDH1/2* and more recently with *TET2* mutations[111], and in patients with complex karyotypes[82]. Comparison of 35 paired diagnosis and relapse samples identified mutation instability and loss of the mutation at first relapse in 34% of cases. This finding suggests that *WT1* mutations do not play a leukemia initiating role, but rather are secondary events[106]. *WT1* mutations result in lowered levels of 5-hydroxymethylcytosine and reduced TET2 function in AML patients that leads to altered DNA methylation[111]. No specific therapies have

been implicated for patients with *WT1* mutations or *WT1* overexpression to date.

2.8 High-risk AML

AML patients falling into the high-risk stratification group most commonly have monosomies of chromosome 5 or 7, deletions of the long arm of chromosome 3 and complex karyotype (at least three different genomic alterations)[14]. More recently, a distinct subgroup of patients has been identified with monosomal karyotype (MK; two or more monosomies or a single monosomy together with structural changes) that confers extremely poor prognosis and might serve as a better poor prognosis predictor than complex karyotype[112,113]. In fact, patients with noncomplex MK do as poorly as patients with complex MK with median survival of six months[45,113]. Hence, patients in this category could be considered for HSCT and/or investigational therapy. Genomic aberrations involving chromosomes 5 and 7 in the context of complex karyotype are frequently associated with *TP53* deletions, which represent one of the most adverse markers in AML with very poor patient survival[114,115]. In addition, AML with myelodysplastic changes (often termed secondary AML) as well as therapy related (following cytotoxic therapy) AML has unfavorable prognosis, likely as a results of genomic aberrations overlapping with complex and monosomal karyotypes such as *TP53* deletions and loss of chromosomes 5 and 7[37]. AML harboring MLL-fusions can also fall in the high-risk patients group, but the prognostic risk of MLL translocations is linked with the partner gene[82]. For patients with poor prognosis the only potentially curative treatment is HSCT.

Table 2. *Overall risk assessment of AML in light of integrated genetic analysis*

	Overall risk			**Probability of overall survival**
Cytogenetic risk	Good	Intermediate	Poor	
Favorable	*PML-RARα*			60%
	CBF leukemia			
	biallelic CEBPα			
Intermediate (normal karyotype)	Mutant *NPM1* and *IDH1/2* *FLT3*-ITD -			80-90%
		wt *ASXL1* *FLT3*-ITD -		40%
		wt *MLL*-PTD *FLT3*-ITD -		
		wt *PHF6* *FLT3*-ITD -		
		wt *TET2* *FLT3*-ITD -		
		Mutant *CEBPα* *FLT3*-ITD – or +		
		wt *MLL*-PTD *FLT3*-ITD +		
		wt *TET2* *FLT3*-ITD +		
		wt *DNMT3α* *FLT3*-ITD +		
			Mutant *TET2* *FLT3*-ITD -	10-15%
			Mutant *MLL*-PTD *FLT3*-ITD -	
			Mutant *ASXL1* *FLT3*-ITD -	
			Mutant *PHF6* *FLT3*-ITD -	
			Mutant *TET2* *FLT3*-ITD +	
			Mutant *MLL*-PTD *FLT3*-ITD +	
			Mutant *DNMT3α* *FLT3*-ITD +	
			wt *CEBPα* *FLT3*-ITD +	
Unfavorable			*TP53*	0-5%
			Monosomy 7	
			Monosomy 5	
			5q deletion	
			Inv(3) or t(3;3)	
			Monosomal karyotype	
			Complex karyotype	

PTD: partial tandem duplication; wt: wild type. Adapted from Patel et al, N Eng J Med, 2012[82].

3. Genomic landscapes and clonal evolution of AML

The genetic architecture of AML is relatively simpler than that of solid tumors with lesser genomic instability and an average rate of 13 mutations per patient[76]. Previous research has shown that individual hematopoietic stem cells harbor comparable number of mutations to the one detected in *de novo* AML patients, indicating that AML development occurs randomly in a cell that accidentally accumulates transforming combination of mutations[116]. The number of driver mutations that are required for a malignant transformation is not well defined, but the working hypothesis is that there are 5-7 driver mutations in epithelial cancers whereas that number may be lower in hematologic malignancies[117]. The recurrent mutations in AML are reasonably well outlined and grouped into functional classes (Figure 4).

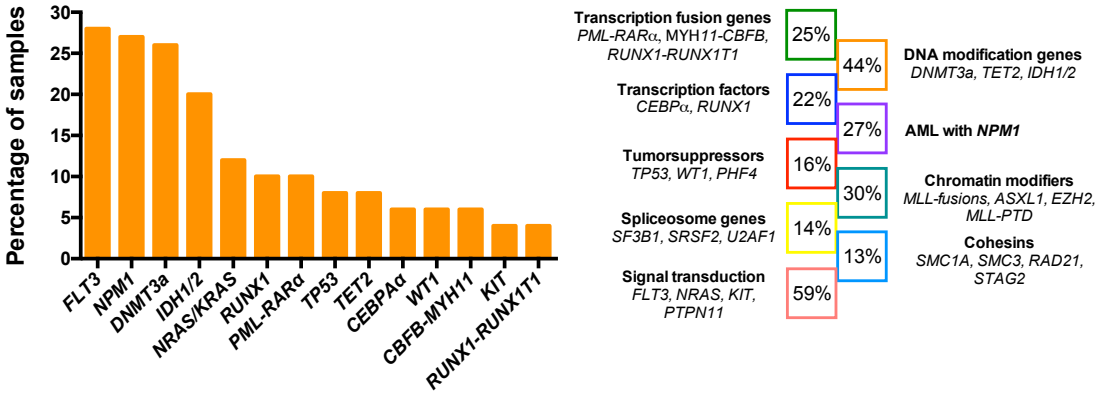


Figure 4. *Percentage of significantly mutated genes and recurrent genomic aberrations in AML out of 200 patient samples evaluated in the AML TCGA study[76].*

Based on the two-hit model of leukemia pathogenesis, briefly described above, AML initiating events are thought to be class II mutations (ones affecting transcription and cell differentiation; e.g. PML-RARα or MLL-fusions) with class I mutations (ones conferring proliferative advantage) occurring later in disease progression[48,117]. Co-occurrence and exclusivity analysis shows that certain mutations are synergistic whereas others are redundant, giving insights into the biology of AML. Studies in mice suggest that only two mutations are sufficient for AML initiation[118,119] and this is corroborated in human genome sequencing studies with numerous AML patients harboring one or two identifiable driver mutations. While two driver mutations may be sufficient for leukemic transformation, the majority of patients carry three or more drivers at clinical presentation[116].

Two distinct concepts of cancer evolution have been proposed: linear and branching evolution (Figure 5). Sequential dominant clones arising as a result of step-wise acquisition of driver mutations define linear evolution. However, deep sequencing studies have shown that AML genomes are highly mutationally complex and variability and dominance of clones alter during the disease course[120,121]. Hence, due to the significant increase of genetic and clonal heterogeneity and the continuous accumulation of mutations it is likely that majority of tumors undergo branching evolution[117]. In AML majority of

disease-related mutations are common to all clones as the initiating event occurs in a cells with mutational history, whereas subclonal mutations account for 14% of total mutations[116]. Evidence exists that both linear and branching clonal evolution occurs in AML. In some cases only one mutation cluster is detected in the primary tumor that gains additional mutations at relapse in line with linear clonal architecture. In contrast, in other cases several mutation clusters are present in the primary and only certain subclones that evade therapy extinction survive and expand at relapse (branching clonal architecture)[121]. Moreover, several studies have shown that disease relapse in AML occurs as a result of re-emergence or evolution of the founding clone[121-123], indicating that the genetic heterogeneity of leukemic stem cells at diagnosis is the central problem. Questions arise whether combination of targeted therapies as first line treatment of AML will deliver improved outcomes, but success will likely depend on ability to target all clones and prevention of further clonal evolution that commonly occurs in response to therapy[121].

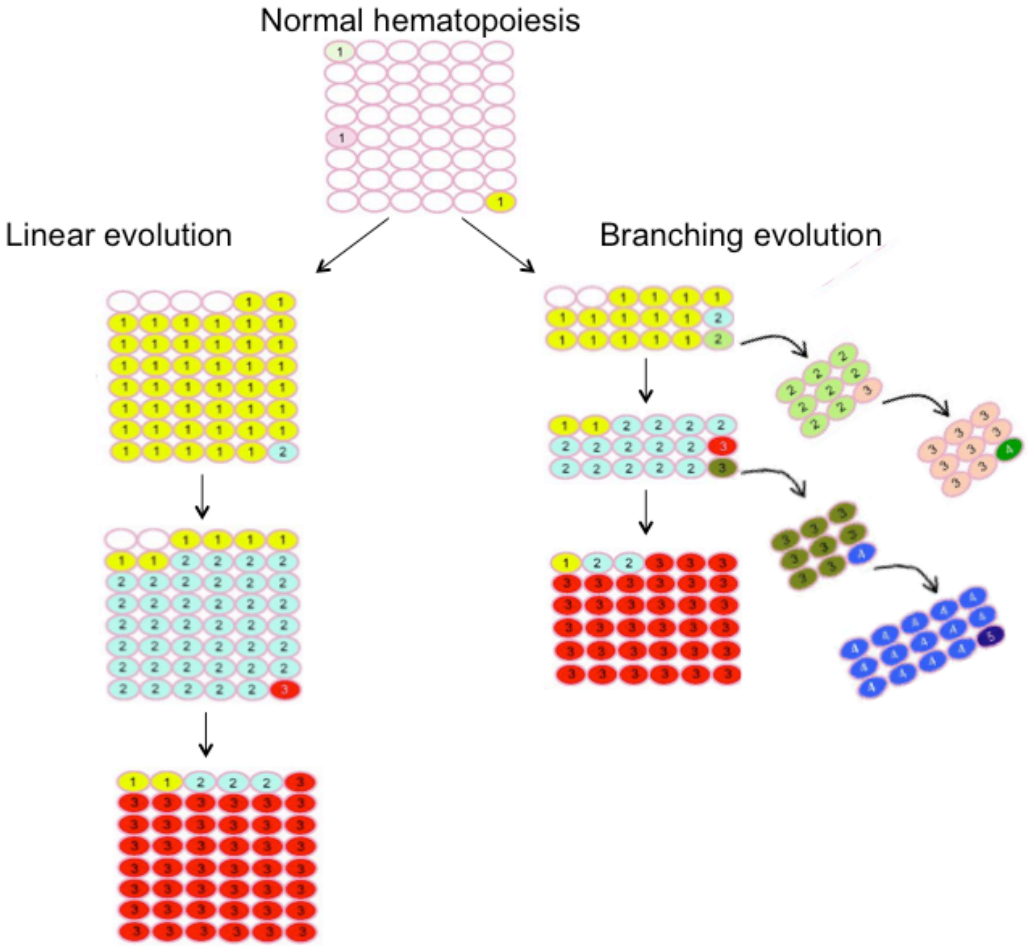


Figure 5. *Graphical representation of linear and branching clonal evolution. Adapted from Grove and Vassiliou, Disease Models & Mechanisms, 2014*[117].

4. Further therapeutic perspectives in AML

While most AML patients can be comprehensively characterized based on cytogenetics and molecular markers, this has still not led to significant therapeutic advances. The cornerstone of AML treatment still remains

unspecific cytotoxic therapy with the exception of APL. Given the dismal outcome of refractory and older patients, novel therapies are urgently needed. Evidence exists that RTKs have a significant role in leukemogenesis and can serve as important targets for molecularly targeted therapy (Figure 6)[124]. One example of this is the development of FLT3 specific inhibitors, which are explored in clinical trials as monotherapy or in combination with conventional chemotherapy. Promising first in human study results have been observed for the second-generation inhibitor quizartinib in relapsed and refractory patients with *FLT3*-ITDs where 10 out 18 (56%) patients had a clinical response with median response duration and overall survival of 13.3 and 14 weeks, respectively[125]. Moreover, phase II trials gave additional proof of quizartinib's efficacy as a single agent in *FLT3*-ITD positive AML[126]. Quizartinib acts by potent and selective inhibition of FLT3 resulting in rapid clearance of peripheral blasts and initiation of apoptosis in majority of *FLT3*-ITD driven AML. On the contrary, the effects in the bone marrow are typically not cytotoxic, but rather terminal differentiation initiating that is supported by flow cytometric analysis revealing increased expression of monocytic markers (CD15) and reduced expression of CD34 and CD117[127]. Several other FLT3 inhibitors are at various stages of clinical development for treatment of AML such as sorafenib[128,129] (currently in phase II in combinatorial setting), midostaurin[130] (currently in phase III as single agent or combinatorial therapy), and crenolanib[131,132] (currently in phase II; NCT01657682). Clinical challenges pertaining to FLT3 inhibitors are maintenance of an effective plasma concentration, lack of efficacy against both *FLT3*-ITDs and *FLT3*-TKD, QTc prolongation, and selection for resistant mutations[133]. It remains to be seen whether FLT3 specific inhibitors will reach the market and have an impact on the outcome of AML patients, especially for those with *FLT3* mutations.

As mentioned previously, *KIT* mutations are present in significant proportion of CBF AML patients. Hence, targeting the resulting RTK CD117 expressed on hematopoietic progenitor cells is an attractive strategy for this patient population. KIT signaling is involved in the regulation of proliferation, differentiation and apoptosis. Candidate agents are inhibitors of Abelson murine leukemia viral oncogene homolog 1 (ABL1), imatinib and dasatinib, which also target KIT with dasatinib currently explored in trials in combination with chemotherapy (NCT01876953)[134]. Moreover, aberrant activation of the MAPK pathway has been implicated in leukemogenesis[135]. The pathway is frequently deregulated in many cancer types including AML, as it plays a crucial role in regulation of cell proliferation. Mutations in *NRAS* and *KRAS* occur in 12-15% of all AML cases, but are most common in NK AML[76]. Several different agents exist with capabilities to in theory block the RAS/RAF/MEK/ERK cascade, however single agent approaches towards RAS-driven cancers including AML have not been successful and therefore tailored and more personalized combinatorial strategies would be needed to target those, likely involving specific RAF, MEK or ERK inhibitors as backbones. The MEK inhibitor selumetinib has been explored in phase I and II trials with modest single-agent efficacy and good safety profile[136,137], indicating that it can be explored further for combinatorial approaches. Previous research has shown that the PI3K/AKT/mTOR[138] and the JAK/STAT3/5[139] pathways are frequently activated in AML[135]. Nonetheless,

there is little evidence that inhibiting these pathways with single agents would be an efficient strategy. While there is an indication that molecularly targeted therapy could have a distinct clinical implication for AML patients, the challenge remains in broadening the understanding of AML pathogenesis and identifying effective combinatorial regimens and subgroups of patients likely to benefit.

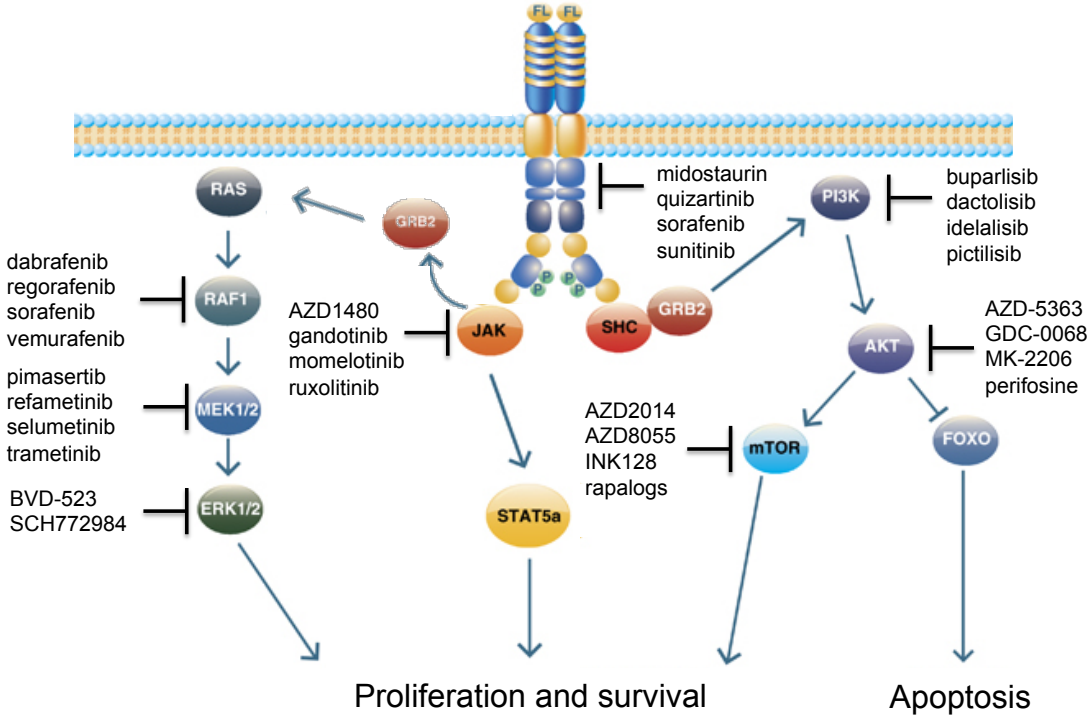


***Figure 6**. Schematic representation of the FLT3 signaling pathway and potential points of inhibition with approved and emerging small molecule inhibitors. Adapted from Swords et al, Leukemia, 2012[140].*

5. CML

CML is a clonal hematopoietic stem cell malignancy affecting approximately one in 100,000 individuals and accounting for 15% of all new leukemia cases in Western countries. In line with AML, the disease is slightly more common in males than in females[15,141] and the median age of diagnosis is 64[142]. BCR-ABL1 is a unique diagnosis and disease monitoring biomarker for CML, which is amenable to drug targeting by ABL1 specific tyrosine kinase inhibitors. Diagnosis confirmation is achieved by detection of the Philadelphia chromosome and BCR-ABL1 transcripts in peripheral blood or bone marrow with chromosome banding analysis, FISH cytogenetics and/or quantitative PCR[142].

Three different disease stages have been recognized in CML according to clinical features and laboratory findings, with majority of patients presenting in the chronic phase. Without adequate medical intervention, the disease progresses to an accelerated phase and eventually to an acute-leukemia like disease known as blast crisis within 3-5 years[141]. In chronic phase the myeloid lineage is expanded but cell differentiation is preserved and patients are generally asymptomatic or present with mild fatigue, anemia, splenomegaly,

and/or weight loss[143]. Patients are increasingly being diagnosed by serendipitous finding of elevated WBC count in routine heath screening[144]. Accelerated phase and blast crisis (Figure 7) are largely distinguished on the proportion of blasts present in the blood and bone marrow of a patient with slightly different definitions according to the WHO (10-19% blasts in peripheral blood or bone marrow for AP; ≥20% BC)[10] and European Leukemia Net (ELN; 15-29% blasts for AP; ≥30% for BC)[145] guidelines. The accelerated phase is important as it indicates that the disease is advancing and conversion to blast crisis is forthcoming. Moreover, the efficacy of drug treatment is reduced in patients with advanced disease. Besides the increasing blast count, blast crisis CML is defined by extramedullary proliferation of blasts, large blast foci in the bone marrow or spleen, and complete blockade of terminal differentiation[141]. Moreover, the progression to blast crisis CML is associated with the gain of additional mutations[146].

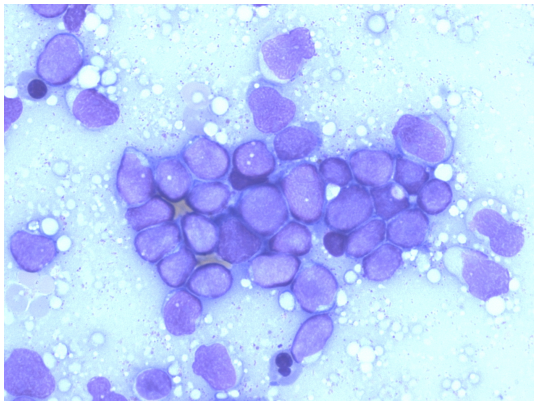


Figure 7*. CML cells in lymphatic blast crisis in bone marrow. Courtesy of Satu Mustjoki.*

5.1 *BCR-ABL1*

The first chromosomal aberration identified in cancer was the Philadelphia chromosome[147]. The Philadelphia chromosome is a shortened chromosome 22 arising as a result of a reciprocal translocation of the *ABL1* gene on chromosome 9 and breakpoint cluster (*BCR*) gene on chromosome 22 (Figure 8). This translocation is detected in over 95% of CML patients, 25-30% of acute lymphoblastic leukemia (ALL) patients and very rarely in AML. The *ABL1* gene is juxtaposed to the proximal part of the *BCR* gene, giving rise to an abnormal fusion gene BCR-ABL1 that encodes for a tyrosine kinase with constitutive activity essential for the oncogenic aptitude of BCR-ABL1[148]. BCR-ABL1 signals via numerous pathways such as the MAPK, PI3K/AKT, and JAK/STAT to enhance cellular proliferation, modify cell differentiation and prevent apoptosis (Figure 9)[149]. The breakpoints of the fusion vary ensuing various DNA amounts of *BCR* to be fused with *ABL1* (exons 2-11). In CML the most common break position is between exons 12 and 16 known as the major breakpoint cluster region (M-bcr). However, in minor proportion of patients the disruption occurs more distally (between exons 19 and 20). On the other hand, in Philadelphia chromosome-positive (Ph+) ALL half of the patients have breaks within M-bcr and the other half have breaks distal to the first exon of *BCR*. Therefore, BCR-ABL1 proteins can range from 185 to 230 kDa

with the smallest containing less of BCR than the others, but all comprise of equal amount of ABL1[148,150]. The p210 BCR-ABL1 is found in almost all Ph+ CML patients and 30-35% of Ph+ ALL patients.

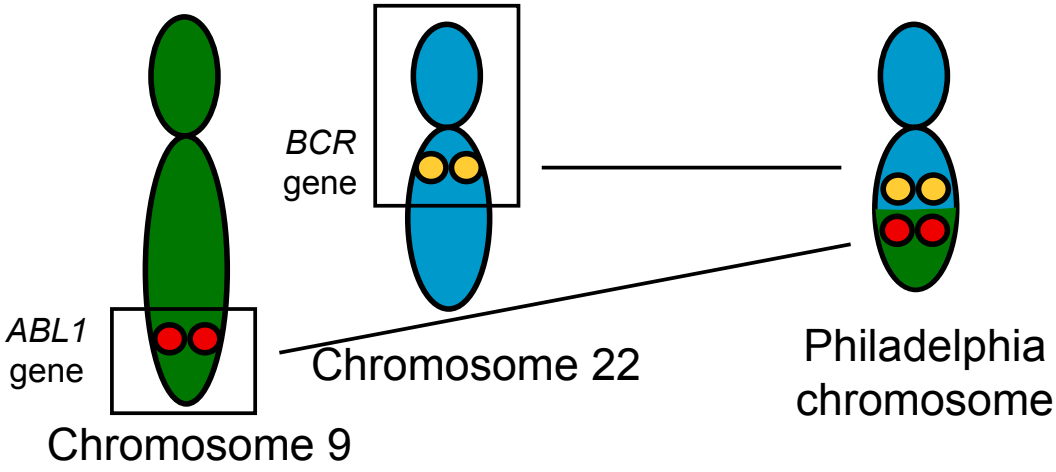


***Figure 8**. Schematic representation of the formation of the Philadelphia chromosome.*

Human ABL1 has a molecular weight of 145-kDa and is ubiquitously expressed. However, in hematopoietic cells its levels decrease with myeloid maturation[151]. ABL1 is a non-receptor tyrosine kinase with variable biological functions. With its SH1 domain it phosphorylates substrate proteins thereby influencing key cellular activities such as increased proliferation, loss of stromal adhesion and resistance of apoptosis[141]. Moreover, ABL1 shuttles between the nucleus (has DNA binding ability) and the cytoplasm (binds to actin cytoskeleton)[152], but in hematopoietic cells ABL1 is more commonly cytoplasmic[151]. While cytoplasmic ABL1 is involved in signaling and cytoskeletal molding, its functions in the nucleus have been connected to regulation of the cell cycle[152] and genotoxicity[153]. The phosphorylation of ABL1 is strictly regulated most likely by motifs in the N-terminus, which are lost in the formation of BCR-ABL1 resulting in the constitutive kinase activity. Similarly, BCR travels between the nucleus and cytoplasm and its expression is reduced with myeloid maturation in hematopoietic cells[154]. The *BCR* gene contains many diverse functional motifs and in eukaryotes it plays a role in two main signaling processes[155,156], phosphorylation and guanosine triphosphate (GTP) binding[132,133]. The first exon of *BCR* is crucial for oncogenic transformation as it is the only exon that is present in all known BCR-ABL1 fusion proteins. Moreover, the first exon has serine and threonine kinase actiivity with ability to phosphorylate itself and main substrates thereby transmitting cellular signals. There are also a number of Src-homology-2 (SH2) binding domains in the first exon, which are critical for association of singnal transduction complexes[148].

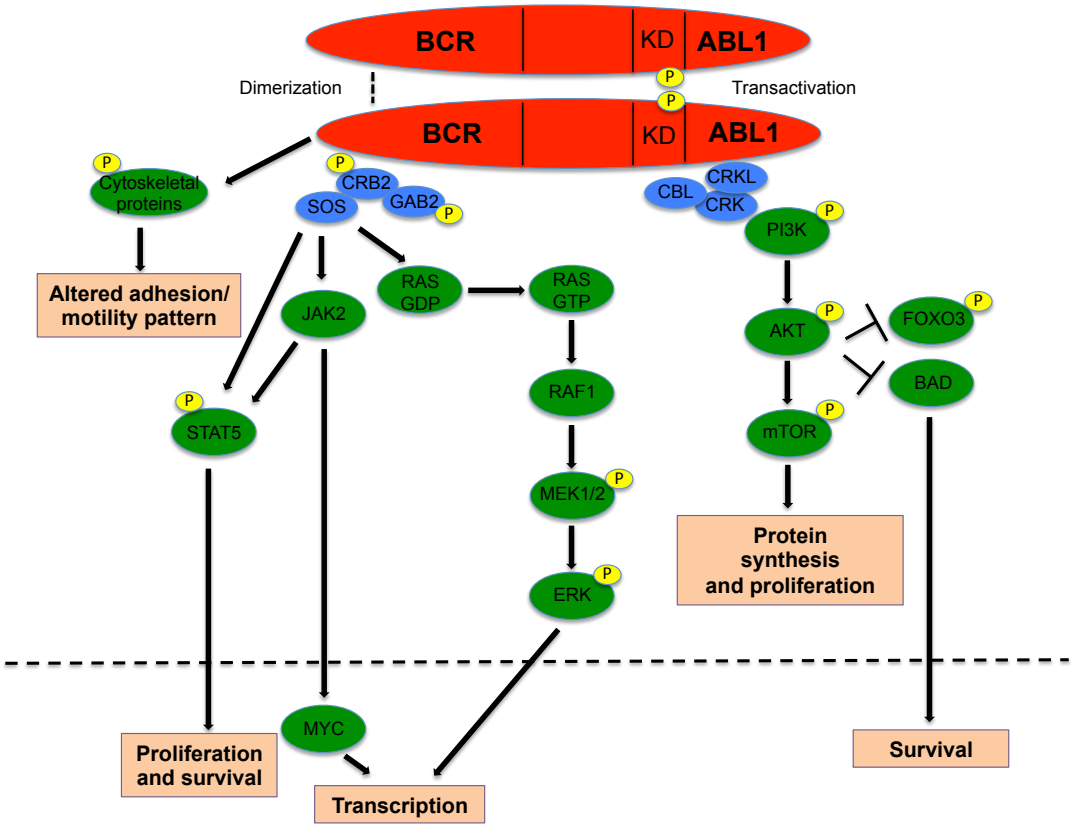


***Figure 9**. A simplified cartoon of the BCR-ABL1 signaling network. BCR-ABL1 dimerization initiates autophosphorylation and activation of the kinase and creates docking sites for adapter proteins (blue), which play a role in activation of downstream effector signals (green) that cummulatively lead to increased survival, block of appoptosis and changes in cell adhesion and migration. Adapted from O'Hare et al, Clin Cancer Res, 2011[157].*

***Table 3**. Biological features of BCR-ABL1 protein variants*

	p190 BCR-ABL1	p210 BCR-ABL1
Constitutive tyrosine kinase activity	✓	✓
Programed cell death attenuation	✓	✓
Associated with JAK/STAT5 signaling	✓	✓
Associated with PI3K/AKT signaling	✓	✓
Transforming activity partially mediated by RAS signaling	✓	✓
May induce alterations in adhesion properties	✓	✓
Induces ubiquitin-mediated degradation of Abelson interacting protein	✓	
Cytoskeletal actin binding		✓
Activates Jun kinase		✓
Interacts with and regulates DNA repair proteins		✓
Interacts with KIT and IL-3 receptors		✓
Upregulates production of IL-3		✓

Modified from a review by Kurzrock et al, Ann Intern Med, 2003[148].

The level of oncogenic potential of BCR-ABL1 correlates with the level of tyrosine kinase activity[158]. Hence, the p190 BCR-ABL1 is linked with the development of more aggressive acute leukemia phenotype as it has the

highest tyrosine kinase activity, whereas the p210 BCR-ABL1 is responsible for the initiation of more indolent chronic leukemia phenotype[148]. The diverse biological features of the two protein variants of BCR-ABL1 are summarized in Table 3.

5.2 Therapy and outcome of CML patients

Prior to 1998, therapy for CML was limited to interferon α, busulfan, hydroxyurea, and HSCT with median patient survival of 5-7 years. Even though, these therapies have shown efficacy in patients with CML, their use is linked with adverse effects that have unfavorable impact on quality of life. Interferon α treatment could lead to complete hematologic remission, whereas only in a fraction of patients complete cytogenetic remission. In 1996, Brian Druker and colleagues discovered a specific ABL1 tyrosine kinase inhibitor (STI571; imatinib) and reported efficacy in CML cell lines *in vitro*[159]. First-in-human studies in accelerated phase patients showed that imatinib controlled blood counts, restored chronic phase and induced cytogenetic responses in significant proportion of patients[160]. Moreover, in a phase II study imatinib exhibited high incidence of complete cytogenetic remission in chronic phase patients that had previously failed interferon α[161]. Imatinib became the first line treatment for CML patients following the IRIS study[162], which compared imatinib vs. low dose cytarabine and interferon α in newly diagnosed patients. Imatinib has since revolutionized treatment, management and outcome of the disease and has served as an exemplar for molecularly targeted agents for cancer therapy. In less than 10 years, the prognosis of the disease has improved from deadly to 80-90% of patients being alive at 10 years[163].

The anti-leukemia effect of imatinib is achieved by competitive inhibition of the ATP binding site in the kinase domain of ABL1, thus blocking BCR-ABL1 authophosphorylation[164]. Imatinib is able to bind to the ABL1 kinase domain only when the conformation of activation loop of the kinase is in an inactive state (DFG-out)[165]. Besides ABL1, imatinib also potently inhibits KIT, colony-stimulating factor-1 receptor (CSF1R), and platelet derived growth factor receptor (PDGFR) α and β[166]. The five year follow up of the IRIS study showed that complete hematologic response (normalization of blood cell counts), major cytogenetic response (0-35% of Ph+ metaphases), and complete cytogenetic response (0% Ph+ metaphases) was achieved in 98%, 92%, and 87% of imatinib treated patients, respectively[167]. Moreover, 8-year follow up revealed that event and progression free survival in patients treated with imatinib was 81% and 92%, respectively[168]. More recent data have demonstrated that acquisition of deeper molecular responses over time is strongly associated with progression-free survival[169] and BCR-ABL1 transcript levels of less than 10% at 3 months forecasts for complete cytogenetic response, overall and progression-free survival[170]. Imatinib is generally well tolerated and the most common side effects include myelosuppression, edema, nausea, diarrhea, headaches, itchy rash, and myalgia[142].

5.3 Imatinib resistance

Although treatment with imatinib leads to high response rates, still approximately one third of patients require alternative therapy due to primary resistance (lack of efficacy), acquired resistance (loss of efficacy) and/or intolerance. Several resistance mechanisms have been recognized such as BCR-ABL1 amplification, overexpression of the multidrug resistant P-glycoprotein, minimal activity of the drug influx transporter OCT1, and the occurrence of point mutations in the ABL1 kinase domain[171-174]. The manifestation of point mutations is the most common resistance mechanism, which interferes with the inhibitor-binding site or leads to stabilization of BCR-ABL1 in a conformation with lower binding affinity for imatinib. Point mutations in the kinase domain were initially identified in 11 patients with advance phase disease resistant to imatinib[173]. In six of those patients, the same threonine to isoleucine amino acid substitution at position 315 was detected, known as the T315I gatekeeper mutation (Figure 10). Since then, over 100 different nucleotide changes have been reported with 15 amino acid substitutions accounting for more than 85% of mutations seen in the clinic[141]. Imatinib resistance led to the development of second and third generation ABL1 specific inhibitors, each with diverse potency, specificity and mutation coverage spectra. Currently there are four different ABL1 TKIs on the market in addition to imatinib: dasatinib, nilotinib, bosutinib and ponatinib. The T315I mutation occurs in 20% of relapsed and refractory patients and confers resistance to all approved BCR-ABL1 inhibitors, except ponatinib. Even though the T315I mutation does not destabilize the overall structure of BCR-ABL1, it has an effect on the topology of the ATP binding side[175]. Lack of efficacy of first and second-generation inhibitors in this setting occurs due to either elimination of the hydroxyl group critical for hydrogen bonding with the isoleucine substitution or steric hindrance that prevents TKI binding to the hydrophobic pocket whilst allowing access to ATP[175-177].

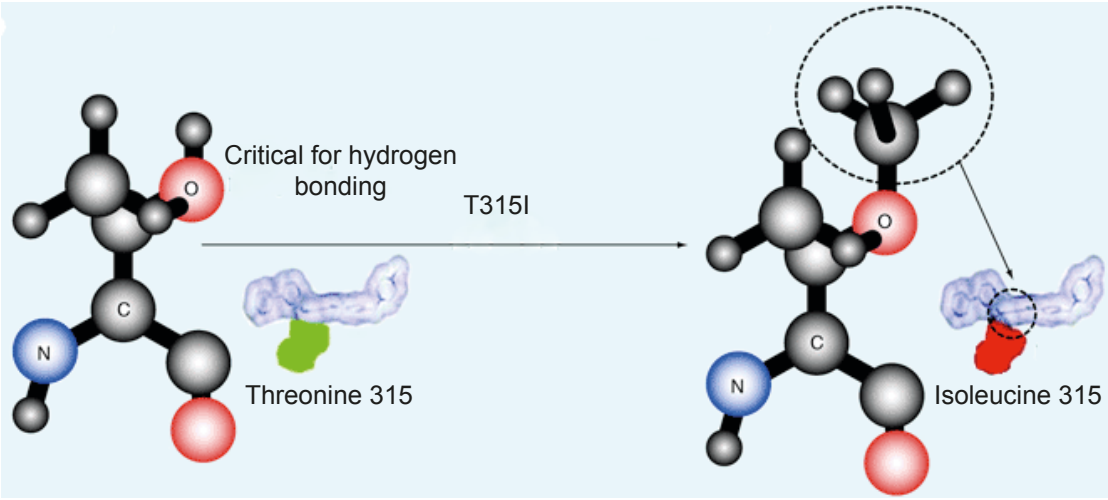


***Figure 10**. Graphical representation of the threonine to isoleucine substitution at residue 315 preventing imatinib to bind to the ATP binding pocket of ABL1. This amino acid change removes a vital oxygen molecule required for hydrogen bonding between imatinib and ABL1. Adapted from Tanaka and Kimura, Expert Rev Anticancer Ther, 2008[178].*

5.4 Dasatinib

Dasatinib, developed by Bristol-Myers Squibb, was the first second generation TKI to exhibit clinical efficacy in imatinib resistant patients[179-181]. Preclinical evaluation showed that dasatinib is 300-fold more potent than imatinib and has inhibitory efficacy against majority of imatinib resistant mutations with noteworthy exception of T315I[182,183]. Besides T315I, dasatinib has lack of activity towards F317V/L, T315A and V299L. In contrast to imatinib, dasatinib can bind to ABL1 when the kinase domain is in an active (DFG-in) or inactive conformation with preference for the active state of the kinase[184]. This feature of dasatinib poses less stringent binding requirements making it less amenable to mutational escape[185]. In addition to ABL1, dasatinib has potent activity towards SRC family kinases, KIT, ephrin receptors, PDGFR-β, among others. Dasatinib was initially approved for CML and Ph+ ALL patients resistant or intolerant to prior therapy including imatinib, but has since been approved for first line treatment of chronic phase CML patients. The most frequently occurring side effects are myelosuppression, pleural effusions, intracranial bleed, QT prolongation, diarrhea, and headache[142].

5.5 Nilotinib

Nilotinib was rationally designed by Novartis to structurally resemble imatinib with improvements in binding affinity, ABL1 selectivity and potency[183]. Therefore, nilotinib is approximately 30-fold more potent than imatinib. Similarly to imatinib, nilotinib binds ABL1 in its inactive conformation, but its superior topographic fit contributes to its mutation coverage[186,187]. In line with dasatinib, nilotinib has activity against most imatinib resistant mutations with the exception of T315I, E225K/V and Y253H[188,189]. Moreover, nilotinib has inhibitory activity against PDGFR, KIT, DDR1, and ephrin receptors. Clinical efficacy of nilotinib was initially demonstrated in imatinib resistant or intolerant patients[190-192], which led to regulatory approval for CML patients in chronic or accelerated phase refractory to prior therapy. Nilotinib has since also been approved for newly diagnosed chronic phase CML patients. The most common side effects of nilotinib are skin rashes, electrolyte imbalance, hyperglycemia, pancreatitis, myelosuppression, fatigue, diarrhea and headache[142].

6.6 Bosutinib

Bosutinib, developed by Pfizer, is a dual ABL1/SRC orally available second generation inhibitor with activity against most imatinib resistant mutants excluding T315I and V299L. Intriguingly, bosutinib has the ability to bind ABL1 in both its active and inactive conformations[193]. Bosutinib is currently approved for CML patients resistant or intolerant to previous therapy as it exhibited efficacy in patients who have lost sensitivity to imatinib, nilotinib and dasatinib[194,195]. Main adverse events of bosutinib treatment are diarrhea and hepatotoxicity, but myelosuppression, nausea, vomiting and skin rashes can also occur[142].

6.7 Ponatinib

The third generation inhibitor ponatinib was rationally designed to overcome the limitations of second-generation inhibitors in targeting the BCR-ABL1(T315I) mutation[196]. Hence, ponatinib is the only clinically available inhibitor to display efficacy against the T315I mutation in addition to most imatinib-resistant mutants[197,198]. Interestingly, a recent study reported that the T315M mutation derived from T315I is associated with high-level ponatinib resistance[199]. Ponatinib binds ABL1 in its inactive conformation and its structure is characterized by a unique triple bond ethynyl linker that effectively overcomes the steric hindrance otherwise caused by I315. Ponatinib is potent inhibitor of a number of other kinases such as VEGFR, PDGFR, FGFR, SRC, KIT, RET and FLT3[197]. Following the pivotal phase II (PACE) trial[200], ponatinib obtained regulatory approval under the FDA's accelerated approval program for CML and Ph+ ALL patients resistant or intolerant to prior tyrosine kinase inhibitor treatment. Responses to ponatinib are durable in chronic phase CML, but typically transient in blast phase CML and Ph+ ALL[200]. In addition, during the EPIC, phase III, trial high incidence of life-threatening blood clots and severe narrowing of blood vessels was observed that led the FDA to suspend the trial and temporarily withdraw ponatinib from the market[201,202]. These side effects most likely result due to ponatinib's potent nature and pan-activity[203]. The drug has since been reinstated for a narrower patient population (adults with CML and Ph+ ALL with T315I mutations or CML and Ph+ ALL patients ineligible for other TKI) with a black box warning[204].

5.8 Sequential TKI treatment and resistance

Newly diagnosed CML patients have the possibility of starting treatment with any of the frontline approved drugs (e.g. imatinib, dasatinib or nilotinib). However, evidence has shown that over 30% of patients will require alternative treatment due to resistance or intolerance of first line TKI[205]. Primary resistance likely occurs due to the presence of kinase domain mutations at baseline. On the other hand, secondary resistance is the result of selection of resistant clones from prolonged exposure to TKIs, which is supported by the observation that sequential TKI treatment frequently promotes greater TKI resistance[206,207]. Kinase domain mutations contributing to drug resistance generally occur at strategic positions (e.g. ATP-binding and activation loops), which block inhibitor binding while maintaining ATP binding ability and kinase activity[208]. Though, some mutations are known to reduce the inherent kinase activity such as the M351T mutation[209]. Mutations can occur in the kinase active site resulting in steric hindrance and at positions that either influence the overall structure of the kinase or preclude the adoption of the inactive state[203].

Previous research has shown that sequential TKI treatment also leads to the development of multiple mutations in the ABL1 kinase domain, which can either be compound (multiple mutations in the same BCR-ABL1 molecule) or polyclonal (multiple mutations occurring in several BCR-ABL1 molecules)[207,210]. In a recent study of 1,700 patient samples, multiple mutations were detected in 11.4% of the cases with 70.2% accounting for

compound and 29.8% for polyclonal mutations[189]. Majority of compound mutations consisted of two mutations (76%) in comparison to triple (21%) and quadruple (3%) mutations. The composition of the compound mutations was in line with TKI treatment history with one or more mutational partners linked with the typical clinical resistance profile of the particular TKI[189]. In addition, two other studies have identified compound and polyclonal mutations that confer clinical resistance to ponatinib with T315I-comprising compound mutants resulting in enhanced resistance to all clinically available TKIs, including ponatinib[199,211]. Interestingly, the compound mutations displayed several-fold higher resistance profile than either of the mutations alone. Moreover, most compound mutations detected in patients so far consist of key single mutations, indicating that there is a restricted number of catalytically sustainable combinations[199]. Nevertheless, the development of complex polymutants represents a novel escape route for resistant clones, warranting robust sequencing methods and rational treatment selection to boost the clinical outcome of Ph+ patients.

6. Ph+ ALL

Even though BCR-ABL1 is sufficient for CML initiation, additional genetic aberrations or transforming events are required for development of Ph+ ALL. Activation of SRC family of kinases[212], deletions in *IKZF1* (IKAROS)[213-216] and *CDKN2A/B*[203,204], and deletions, fusions or amplifications in *PAX5*[217-220] have all been implicated in Ph+ ALL pathogenesis. Ph+ ALL is derived from expansion of immature lymphoid lineage cells. However, a functional study has shown that both CML and Ph+ ALL arise from common cells of origin resembling long-term hematopoietic stem cells (LT-HSCs) [221]. In addition, the study showed that the difference among the disease states occurs during disease-maintenance where STAT5 signaling is essential for CML transformation while IL-7 signaling is critical for LT-HSCs differentiation to pro-B cells and consequent Ph+ ALL initiation[221].

In most Ph+ ALL patients the 190 kDa BCR-ABL1 is expressed, whereas the rest harbor the 210 kDa variant. Several other differences exist between CML and Ph+ ALL at the clinical and molecular level. For instance, the presence of the Philadelphia chromosome is an adverse prognostic factor in ALL, which is not the case in CML. Moreover, Ph+ ALL is characterized with additional epigenetic modifications, copy number alterations, and mutations downstream of BCR-ABL1 that play a role in the aggressive clinical course of the disease, which may include involvement of the central nervous system (CNS). Therefore, preventing leukemia cell growth is not accomplished by merely inhibiting BCR-ABL1[222].

Historically, patients have been treated with aggressive chemotherapy regimens and complete response rates were achieved in 45%-90% of patients. Nevertheless, majority of patients relapse and only few achieve long-term survival. The only curative treatment is allogeneic HSCT for eligible patients (donor is found and patient is able to tolerate the procedure), although relapse following HSCT and treatment-related mortality are not uncommon[223,224]. Nowadays, imatinib has been incorporated into first-line

regimens as its use improves the outcome of Ph+ ALL patients. However, imatinib treatment is susceptible to resistance in this setting as well[225,226]. Dasatinib has been approved as a second-line therapy for Ph+ ALL patients resistant or intolerant to prior therapy, including imatinib. Despite its clinical efficacy, the inhibitory effect against SRK family of kinases[227] and improved CNS penetration[228] than imatinib make it an attractive alternative. In addition to imatinib and dasatinib, ponatinib has regulatory approval as second or third line treatment in patients with Ph+ ALL. Nonetheless, allogeneic HSCT in first complete response should be contemplated for eligible patients[224].

7. Drug sensitivity testing of cancer cells

For a number of decades, efforts have been directed towards developing technologies that directly assess the sensitivity of patient cancer cells to drugs *in vitro*[229,230]. However, success in predicting cancer cell chemosensitivity in a clinical setting has been limited, as most studies have focused on conventional chemotherapeutics[231-234], responses to which are frequently unselective, challenging to decipher and translate to patient care. More and more evidence is accumulating that kinases are frequently deregulated in human cancers, including leukemias, prompting excitement for kinase specific inhibitors. Therefore, unbiased phenotypic cancer drug sensitivity studies focusing on targeted agents hold promise for identification of links between drug responses and genetic and clinical features. For example one such study reported that 70% of leukemia patient samples display hypersensitivity to one or more kinase inhibitors[235], illustrating that this approach can serve as means to discern cancer kinase pathway dependencies and molecular markers of drug response.

Besides drug efficacy testing in primary cancer cells, many more studies have been conducted on human cancer cell lines that might not always fully recapitulate the heterogeneity and genomic complexity of human cancers. One of the first cancer cell line screening programs was the National Cancer Institute 60 (NCI60) platform[236], covering 60 different cell lines representing nine different cancer histopathological types. The NCI60 platform developed technologies that are the foundation of numerous drug screening programs today, but the weakness of using only 60 cell lines in terms of capturing tumor heterogeneity, genetic diversity and low-frequency responders has become more evident lately[237]. Recent efforts such as the Cancer Cell Line Encyclopedia (CCLE)[238] and Cancer Genome Project (CGP)[239] have focused on characterizing the drug sensitivity profiles of several hundred human cancer cell lines (947 cell lines covering 36 tumor types and 24 drugs, and 727 cell lines 16 tumor types and 138 drugs, respectively). In addition to drug sensitivity profiles, the studies generated genomic profiles of the cell lines that facilitated the identification of genetic, lineage, and gene-expression biomarkers of drug sensitivity. However, these studies revealed very few novel drug sensitivity-biomarker links, possibly due to each cancer type being represented by a handful of cell lines that might not be sufficient to comprehensively detect disease subgroup-specific associations. Nonetheless, high-throughput drug sensitivity testing of cancer cell lines can be an attractive approach to identify novel drug-target links and cancer subtypes

that might benefit from a particular drug. However, results still need to be verified and explored in either patient samples or proof of concept clinical trials, as cell lines do not fully resemble the disease manifestation in patients. Therefore, more biologically and clinically relevant data might be obtained from studying cancer patient samples that encompass not only the genetic complexity, but also intricate signaling networks that drive cancer progression and influence response to therapy.

The pharmacogenomics field has been criticized recently, as comparative analyses of CCLE and CGP data found discrepancies in the drug sensitivities reported albeit no large differences in gene expression profiles[240,241]. These findings shed doubt on the value of drug screening of cancer cell lines as means of developing predictive markers of drug response. The observed differences likely stem from no standardized experimental protocols in terms of cell handling, drug concentration ranges, readouts, and modeling and scoring of dose response curves (Table 4)[240,242]. In contrast, higher degree of accordance was detected when comparing CCLE and GlaxoSmithKline (GSK)[243] data on lapatinib and paclitaxel sensitivity as both studies utilized the same readout (ATP production as a measure of cell viability)[240]. This suggests that the assay has significant impact on the quantification of the drug response. Moreover several other factors can influence experiment outcomes: cell culture conditions, cell line genetic deviation due to passaging, compound handling and storage, and assay conditions. For instance, comparison of different readouts has revealed that ATP-based luminescent assays while very sensitive are susceptible to underestimation of drug potency and efficacy, especially for compounds interfering with DNA synthesis[244].

Primary data outputs of high-throughput drug screening studies are compound dose response curves and associated drug response measures such as half-maximal inhibitory concentration (IC_{50}) and area under the curve (AUC). Since no standard drug response metric has been established, most commonly the IC_{50} is used to evaluate drug sensitivity that has limitations in fully capturing the drug response information. Both, AUC and IC_{50}, estimations depend on drug-cell line/type pair, shape of the curve and drug concentration range tested. In addition, heterogeneous cell populations and intrinsic cellular variability might significantly affect the cumulative drug response measured[242,245]. Intriguingly, certain compounds in the CCLE study could be classified as effective or ineffective depending on which metric is used for analysis[241]. Thus, development of novel scoring methods that can capture the qualitative and quantitative drug sensitivity information are needed.

Table 4. Differences and similarities in experimental protocols used in CCLE and CGP studies

	CCLE	CGP
Cell lines	Commercial vendors, COSMIC annotated	Commercial vendors, COSMIC annotated
Culture medium	RPMI or DMEM + 10% FBS	RPMI or DMEM + 5% FBS + penicillin/steptomycin
Cell seeding density	N/A	70% cell confluence
Compound storage	90% DMSO, 10% water at 2 mM (-20°C)	10 mM aliquots (-80°C)
Cell plating	1536 plates (5 µl final volume 250 cells/well)	96- or 384-well plates
Drug concentration	Serial dilutions (2 mM to 636 nM)	The range of concentrations for each compound is based on prior *in vitro* data
Adherent cells	Plated 12-48 h prior to compound addition after which 72-84 h incubation	Plated 1 day prior compound addition after which 72 h incubation
Suspension cells	N/A	Immediately treated with compounds and incubated for 72 h
Viability measurement	ATP based (CellTiter Glo; Promega)	Resazurin based (Sigma) or florescent nucleic acid stain Syto60 (Invitrogen)
Controls	Negative: Vehicle only cells Positive: MG132	Negative: 8 (96-well) or 32 (384) no cells wells Positive: 16 (96) or 42 (384) drug-free wells
Assay reproducibility	Compounds tested in duplicate, some cell lines assayed several time at different time points (data not shown)	Assays performed at two different sites with matched cell collections (data shown for 3 drugs only)
Drug response curve fit	Decision tree methodology (NIH/NCGC assay guidelines)	Bayesian sigmoid model
Drug sensitivity statistic	EC_{50}, IC_{50}, AUC, A_{max}	IC_{25}, IC_{50}, IC_{75}, IC_{90}, AUC

In CGP resazurin was used for detecting cell viability in suspension cells, whereas, Syto60 for adherent cells that detects total cell counts. AUC-area under the curve; COSMIC-catalogue of somatic mutations in cancer; DMSO-dimethyl sulfoxide; FBS-fetal bovine serum; MG132-proteasome inhibitor effectively killing all cells at 1 µM;. Adapted from Hatzis et al, Cancer Res, 2014[242].

AIMS OF THE STUDY

The overall aim of this doctoral dissertation was to functionally profile leukemia patient cells in terms of molecular vulnerabilities with a drug sensitivity and resistance testing platform in order to identify personalized therapy options.

1. Development and implementation of a predictive *in vitro* and *ex vivo* (high-throughput) drug sensitivity and resistance testing platform for personalized cancer medicine applications for leukemia patient samples

2. Development of a score to quantify drug sensitivity and resistance

3. Understanding and identification of molecular drivers of AML using drug screening data

4. Identification of drug repurposing opportunities for leukemia patients

MATERIALS AND METHODS

8. Study specimens

8.1 Patients

AML, CML and Ph+ ALL patients studied in projects I-II and Supplementary article (SA) I were clinically diagnosed based on FAB or WHO criteria[10]. Samples from these patients were primarily collected at Helsinki University Hospital Comprehensive Cancer Center, Department of Hematology after written informed consent and ethical approval of the Institutional Review Board of the Hospital (No. 239/13/00/2010, 303/13/03/01/2011). The consent form also included the possibility to use DSRT data to guide therapies with approved drugs in an off-label mode in accordance with Finnish legislation. In addition, the studies were performed in agreement with Declaration of Helsinki. Specimens from healthy individuals were collected in an outpatient clinic setting with informed consent. Only freshly processed samples were included in all studies.

Study I evaluated the drug sensitivity and molecular profiles of 28 bone marrow samples from 18 AML and high-risk MDS patients. In addition, 7 bone marrow samples from healthy individuals were analyzed as controls. Nine samples were collected from newly diagnosed patients, whereas 19 from relapsed and/or refractory patients. Skin biopsies from the AML and MDS patients were used as germline controls for exome sequencing data. Study II identified and characterized the tyrosine kinase inhibitor axitinib as a putative novel inhibitor of BCR-ABL1(T315I). For this purpose drug sensitivity profiles of samples derived from 3 CML in blast crisis, 3 Ph+ ALL, 33 AML patients, and 7 healthy donors were compared. Four out of 6 Ph+ samples harbored the T315I mutation. Majority of AML samples included in this study were previously analyzed in study I. SA I incorporated a slightly overlapping cohort with study I and utilized 22 and 4 bone marrow aspirates from 14 primarily relapsed and refractory AML patients and 4 healthy donors, respectively. Four samples (from 4 different patients) were not included in study I. Eight samples were obtained from newly diagnosed patients and the remaining 14 from relapsed and/or refractory patients.

8.2 Sample processing and handling

Bone marrow or peripheral blood aspirates from leukemia patients were subjected to Ficoll centrifugation (Ficoll-Paque PREMIUM; GE Healthcare) in order to isolate the mononuclear cells according to manufacturer's instructions. Mononuclear cells were washed, counted, and maintained in mononuclear cell medium (MCM; PromoCell) containing 0.5 µg/ml gentamicin and 2.5 µg/ml amphotericin B. In study I, one sample from a secondary AML patient, was further separated based on CD34 positivity status with magnetic beads according to manufacturer's instructions (Miltenyi Biotech). If there were excess cells available, they were either stored as pellets at -70 or biobanked in liquid nitrogen in FBS and 10% DMSO for future use.

8.3 Cell lines

Besides patient material, study II and SA I utilized cell line material as well. Engineered Ba/F3 cells analyzed in study II were attained from Oregon Health and Science University and cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin. To further illustrate the applicability of the drug sensitivity score, SA I also used publicly available human cancer-cell line drug sensitivity data from the CCLE[238] resource (479 cell lines tested against 24 oncology compounds).

9. Inhibitors

The oncology compound collection was composed of commercially available approved, investigational and experimental anti-cancer compounds. The collection included most US Food and Drug Administration/European Medicines Agency (FDA/EMA) approved oncology drugs and covered a wide array of molecular targets (Table 5). Inhibitors were dissolved in DMSO or water and stored in desiccators according to manufacturer's instructions. Hundred eighty-seven, 252, and 204 anti-cancer agents were used in study I, II and SA I, respectively. Compounds were acquired from National Cancer Institute Drug Testing Program or purchased from: Active Biochem, Axon Medchem, Cayman Chemical Company, ChemieTek, Enzo Life Sciences, LC Laboratories, Santa Cruz Biotechnology, Selleck, Sequoia Research Products, Sigma-Aldrich, and Tocris Biosciences.

9.1 Drug sensitivity and resistance testing (DSRT)

DSRT was conducted on freshly isolated primary mononuclear cells derived from patients or healthy donors. Compounds were preprinted on tissue culture treated 384-well plates (Corning 3707) with an acoustic liquid handling device (Echo 550; Labcyte Inc.). Each compound was dispensed in five different concentrations (10-fold dilutions) covering a 10,000-fold concentration range (e.g. 1-10,000 nM). Ready-made plates were stored until needed in nitrogen pressurized StoragePods (Roylan Developments Ltd.). Prior to cell addition, compounds were suspended in 5 µl MCM and the plates placed on a shaker for 30 minutes. Single cell suspension of mononuclear cells (10,000 cells/well in 20 µl) was then transferred to every well with a MultiDrop Combi (Thermo Scientific) peristaltic dispenser. The microtiter plates were then incubated for 72 h at 37°C and 5% CO_2. Following the 72 h incubation, cell viability was measured using the CellTiter Glo luminescent assay (Promega) according to manufacturer's instructions with either Molecular Devices Paradigm or BMG Labtech PHERAstar plate readers. Sixteen positive (100 µM benzethonium chloride containing wells) and 16 negative (DMSO only) control wells were included on each plate for data normalization. Dotmatics Browser/Studies software (Dotmatics Ltd.) was used for percentage of survival calculation and dose response curve generation for each drug tested. A four-parameter logistic fit function was used to model the dose response curve defined by minimum and maximum inhibition, slope and the inflection point (IC_{50}/EC_{50}) with the top asymptote fixed to 100 and bottom asymptote floating between

0% and 75%. Hence, compounds exhibiting less than 25% inhibition were pondered inactive.

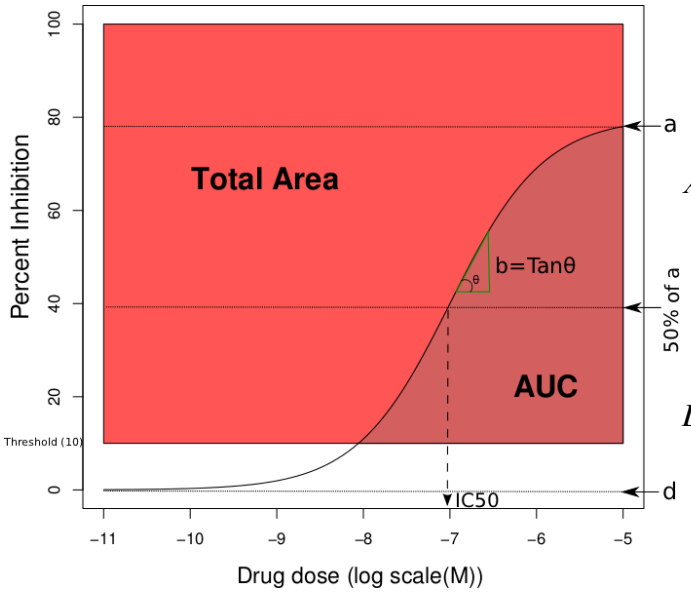
Table 5. Compounds and drug classes included in the DSRT platform

Drug class	Compounds
Apoptotic modulators	AT 101, navitoclax, nutlin-3, obatoclax, serdementan, YM155
Conventional chemotherapeutics	ABT-751, allopurinol, altretamine, amonafide, auranofin, bendamustine, bleomycin, bortezomib, busulfan, camptothecin, capecitabine, carboplatin, carfilzomib, carmustine, chlorambucil, chloroquine, cladribine, clofarabine, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin, docetaxel, doxorubicin, etoposide, floxuridine, fludarabine, fluorouracil, gemcitabine, hydroxyurea, idarubicin, ifosfamide, indibulin, irinotecan, ixabepilone, lomustine, melphalan, mercaptopurine, mitomycin C, mitotane, mitoxantrone, nelarabine, omacetaxine, paclitaxel, patupilone, pentostatin, pipobroman, plicamycin, procarbazine, streptozocin, temozolomide, teniposide, thioTEPA, thioguanine, topotecan, uracil mustard, valrubicin, vinblastine, vincristine, vinorelbine
Differentiating/ epigenetic modifiers	(+)JQ1, arsenic (III) oxide, azacitidine, belinostat, bexarotene, CUDC-907, decitabine, entinostat, iniparib, mocetinostat, niraparib, olaparib, panobinostat, PFI-1, quisinostat, rucaparib, stemregenin 1, tacedinaline, tipifarnib, tretinoin, tubacin, tubastatin A, valproic acid, veliparib, vorinostat, XAV-939
Hormone therapies	4-hydroxytamoxifen, abiraterone, aminoglutethimide, anastrozole, bicalutamide, clomifene, enzalutamide, exemestane, finasteride, flutamide, fulvestant, goserelin, lasofoxifene, letrozole, megestrol, nilutamide, raloxifene, RD162, tamoxifen, toremifene
HSP inhibitors	alvespimycin, BIIB021, geldanamycin, luminespib, tanspimycin, VER 155008
Immunomodulatory agents	bimatoprost, celecoxib, dexamethasone, imiquimod, lenalidomide, levamisole, methylprednisolone, prednisolone, prednisone, thalidomide
Kinase inhibitors	afatinib, alisertib, alvocidib, apitolisib, AT9283, axitinib, AZ 3146, AZD1152-HQPA, AZD1480, AZD4547, AZD7762, AZD8055, BI 2536, binimetinib, BMS-754807, brivanib, bryostatin 1, buparlisib, cabozantinib, canertinib, cediranib, crizotinib, CUDC-101, dactolisib, danusertib, dasatinib, doramapimod, dovitinib, EMD1214063, enzastaurin, erlotinib, fasudil, foretinib, fostamatinib, galunisertib, gandotinib, gefitinib, ibrutinib, idelalisib, imatinib, INK128, KX2-391, lapatinib, lestaurtinib, linifanib, linsitinib, masitinib, MGCD-265, midostaurin, MK-2206, MK-1775, momelotinib, motesanib, neratinib, nilotinib, nintedanib, NVP-BGJ398, omipalisib, OSI-027, palbociclib, pazotinib, perifosine, PF-00477736, PF-04691502, PF-04708671, pictilisib, PIK-75, pilaralisib, pimasertib, ponatinib, quizartinib, rabusertib, RAF265, refametinib, regorafenib, ruboxistaurin, ruxolitinib, saracatinib, seliciclib, selumetinib, SNS-032, sonolisib, sorafenib, sotrastaurin, sunitinib, TAK-733, TAK-901, tamatinib, tandutinib, TGX-221, tivantinib, tivozanib, tofacitinib, trametinib, UCN-01, vandetanib, vatalanib, vemurafenib, volasertib, voxatalisib, VX-11E
Kinesin inhibitors	SB 743921, S-trityl-L-cysteine
Metabolic modifiers	atrovastatin, daporinad, methotrexate, pemetrexed
Rapalogs	everolimus, ridaforolimus, sirolimus, tacrolimus, temsirolimus
Other	15D-PGJ2, 2-methoxyestradiol, anagrelide, erismodegib, fingolimod, galiellalactone, MK-0752, PF-3845, pilocarpine, plerixafor, Prima-1 Met, tarenflurbil, tosedostat, varespladib, vismodegib

9.2 Drug sensitivity score (DSS)

In order to quantify the drug sensitivity data and compare the observed drug responses in different patient samples, a single measure termed drug sensitivity score (DSS) was developed. The DSS takes into account all four curve-fitting parameters, which are used to calculate the AUC in relation to the total area between 10% threshold and 100% inhibition (Figure 11). The score is further normalized by the logarithm of the top asymptote to reduce the impact of toxic drug effects (effect of maximal response at highest tested concentration). The default curve fitting function used in these studies is depicted below where a is top asymptote (max response), b the slope, c the IC_{50} and d the bottom asymptote (min response):

$$y = d + \frac{a - d}{1 + 10^{b(c-x)}}$$



$$AUC = \int_{y(x) \geq T} y(x)\mathrm{d}x = f(a,b,c,d,T)$$

$$DSS = \left\{ \frac{AUC}{TotalArea} \times 100 \right\} / \log_{10}(a)$$

Figure 11. Graphical illustration of the drug sensitivity score calculation. Courtesy of Bhagwan Yadav.

DSS is 0 in cases where the maximum response is 10% or lower or IC_{50} equals or is beyond the maximum drug concentration tested. The possible range of DSS values is between 0 and 50. To identify cancer selective drug responses the DSS values detected in patient samples to ones detected in healthy bone marrow control samples were compared (selective DSS; sDSS). In study I, sDSSs were also utilized to uncover similarities and differences of drug sensitivity profiles and functionally group patient samples and drugs with data clustering analysis (complete-linkage method and Spearman (drugs) and Euclidean (samples) distance measures). In SA I, clustering of drug responses was done with the Ward's algorithm using Spearman (drugs) and Manhattan (samples) distance measures. The DSS-based data analysis platform is freely available as an R-package at: https://dss-calculation.googlecode.com/svn/trunk/).

9.3 Kinase addiction prediction

In order to identify kinase signaling networks involved in disease pathogenesis in the AML patient samples incorporated in study I and SA I, the sDSS data of kinase inhibitors was used. The goal was to predict sample-specific kinase addictions by relating patient sample-specific sDSS responses to target specificity data of 35 kinase inhibitors (overlapping between the studies) derived from a recent comprehensive kinase inhibitor selectivity study[246]. With this data a kinase inhibition sensitivity score (KISS) was developed that predicts how amenable the patient cells are to inhibition of a particular kinase. The KISS entails calculating the mean sDSSs of those agents selectively targeting a kinase of interest. To refine the obtained results, gene expression data was correlated with the candidate kinase addictions to eliminate nonexpressed targets. The expressed kinases were then used to characterize a putative 'kinaddictome' for a particular patient sample and determine the kinase pathway dependence. KISS data was visualized with automated layout options in the Cytoscape software[247].

9.4 CCLE drug screening approach and data

In the CCLE study each compound was tested in 8 different concentrations (2.5, 8, 25, 80, 250, 800, 2,530, and 8,000 nM). Since technical replicate data was available, in SA I DSS was calculated for the median dose response data. For comparative analysis of our AML patient sample drug screening data the DSS as well as Activity Area[238] (AA; metric used in CCLE) calculations were implemented. AA takes into account the difference between the measured response (relative growth inhibition %) and reference response (response set to 0) over eight concentrations. Hence, AA is 0 when no drug efficacy is observed and 8 when there is 100% inhibition at all 8 drug concentrations.

9.5 Engineered Ba/F3 cell proliferation assays

In study II to illustrate that axitinib specifically inhibits BCR-ABL1(T315I) in cells, Ba/F3 transformed to express BCR-ABL1 or BCR-ABL1(T315I) were utilized. Engineered Ba/F3 cells were maintained in RPMI-1640 with 1% FBS and 1% penicillin/streptomycin and seeded in 96-well flat bottom plates at 1,500 cells/well. Imatinib, ponatinib and axitinib were initially dissolved in DMSO to make 10 mM stocks, then 3-fold serial diluted in DMSO in 96-well plates after which 40-fold diluted in complete media to generate a 5X source plate. Twenty-five µl of compound suspension (per well) from the 5X source plate was transferred to the cells containing assay plate and the assay plates were incubated for 96 h at 37°C and 5% CO_2. Prior to reagent addition, 80 µl of supernatant was removed from each assay plate well, following a brief centrifugation step, and the cells were resuspended with 100 µl of fresh medium. Cell viability was measured after 6 h incubation with resazurin (15 µl/well of 1 mg/ml; Sigma) at 530 nm excitation and 595 nm emission wavelengths.

To evaluate axitinib's ability to inhibit the growth of a wider panel of engineered Ba/F3 cells, cells were dispensed in 96-well round bottom plates at concentration of 10,000 cells/well in RPMI-1640 and 10% FBS and increasing concentrations of axitinib (0-10,000 nM). Cells were incubated with drug for 72 h and cell viability was measured with the tritiated thymidine incorporation assay as previously reported by le Coutre and collegues[248]. Data was analyzed with GraphPad Prism software.

10. DNA and RNA isolation

DNA was isolated from bone marrow or peripheral blood mononuclear cells and skin cells with the DNeasy Blood & Tissue Kit (Quigen). RNA was extracted with miRNeasy Kit (Qiagen). Concentration of DNA and RNA were quantified with Nanodrop (Thermo Fischer), Qubit 2.0 (Life Technologies), or Agilent 2100 bioanalyzer (Agilent Technologies).

10.1 DNA sequencing

In study I exome sequencing was performed on 20 and in SA I on 12 AML patients samples. Moreover, whole genome sequencing was done on skin and AML cells derived from sample 784_2. Approximately 3 µg of DNA was fragmented and prepared according to the NEBNext DNA Sample Prep Master Mix protocol (New England Biolabs). The Nimblegen SeqCap EZ v2 capture Kit (Roche NimbleGen) was used for exome capture. DNA sequencing (exomes and genomes) was done with Illumina HiSeq 1500, 2000 or 2500 instruments (Illumina). Forty million base pair paired-end reads were sequenced from each germline control sample and 100 million for each tumor sample. DNA isolated from the leukemic cells of patient 1497 was sequenced on the MiSeq sequencer (Illumina) with the Illumina TruSeq Amplicon Cancer Panel. The sequence data was then processed with bioinformatics analysis pipelines as previously described[249,250]. Cancer somatic mutations were called with the VarScan2 somatic mutation caller[251]. Data annotation was performed with SnpEff (Ensembl v60 annotation database)[252], while filtering with RepeatMasker track (University of California, Santa Cruz genome browser) and Single Nucleotide Polymorphism database for false-positive calls due to genomic repeats and misclassified germline variants. Candidate mutations were further visually verified with the Integrated Genomics Browser (Broad Institute). Variant allele frequencies were used to inspect not high-confidence mutation calls and identify regions of loss of heterozygosity.

10.2 Capillary sequencing

Capillary sequencing was used to determine *FLT3*-ITD mutational status of AML patient samples. Isolated genomic DNA was amplified by qualitative PCR as per Kottaridis et al[253] using a 6-carboxyfluorescein-labeled forward primer. PCR products were size-separated on agarose gel and by capillary electrophoresis using an ABI3500Dx Genetic Analyzer and sequenced via M13-tailed direct sequencing. Minimal residual disease level analysis was performed with real-time quantitative PCR (RT-PCR). Albumin gene levels were used to normalize the RT-PCR data.

10.3 Amplicon sequencing

Somatic mutation data in study I was verified by amplicon sequencing with locus-specific PCR primers containing Illumina compatible adapter and grafting sequences and an amplicon specific index sequence (6 base pairs). For the PCR reaction, 10 ng of DNA were combined with 10 µl 2x Phusion High-Fidelity PCR Master Mix (Thermo Scientific) and 0.5 µM of each primer. Primer sequences are provided in Table 6. Sample purification was done with Performa V3 96-well Short Plate and QuickStep2 SOPE Resin (EdgeBio). Amplicons were sequenced on an Illumina HiSeq2000 (Illumina) as 101-base pair paired-end reads and one 7-base pair index read.

Table 6. Primer sequences for amplicon sequencing

Target	Sequence (5'-3')
PDCD10-F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT CCGATCTAAACAACTAGGCATAAACCAACA
PDCD10-R	CAAGCAGAAGACGGCATACGAGAT[AAGCTA]GTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTCAACAGGGATATAGCTAGTGCAA
BRWD3-F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT CCGATCTTTTTCAAGTCTCCGCCTGAT
BRWD3-R	CAAGCAGAAGACGGCATACGAGAT[TTGACT]GTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTTAGATTTTGCCAGCCCTTTT
CREM-F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT CCGATCTTGCTACCATGGCAGTACCAA
CREM-R	CAAGCAGAAGACGGCATACGAGAT[TACAAG]GTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTGGAACAATTTAATGCCAAAACC
H2AFZ-F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCTGGAATCCAGGCATCCTTTAG
H2AFZ-R	CAAGCAGAAGACGGCATACGAGAT[TCAAGT]GTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTCACTTTCTTGGTTTCAAATACTGTG
PDE6C-F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCTGAAAAATCCTGAATTGTATGAACC
PDE6C-R	CAAGCAGAAGACGGCATACGAGAT[GATCTG]GTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTCGTACCTCATCCTGCAACAG
SDR42E1-F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCTCCTAGCTCTTTCTTGGCTTTCTC
SDR42E1-R	CAAGCAGAAGACGGCATACGAGAT[CTGATC]GTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTGACCTTGGTCTACTGCTTTGC
TP63-F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCTGTGAGGGGCCGTGAGACT
TP63-R	CAAGCAGAAGACGGCATACGAGAT[GGAACT]GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTGCCTCCTAAAATGACACGTTG
WT1_ex6-F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCTCCTGGGTAAGCACACATGAA
WT1_ex6-R	CAAGCAGAAGACGGCATACGAGAT[ATTGGC]GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTGCTTAAAGCCTCCCTTCCTC
WT1_ex8-F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCTTTACCTGTATGAGTCCTGGTGTG
WT1_ex8-R	CAAGCAGAAGACGGCATACGAGAT[GTAGCC]GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTCCTTGTGGGCCTCACTGT

Target specific sequences are underlined and index sequences are in brackets.

10.4 Quantification of BCR-ABL1(T315I) transcript levels

Real-time quantitative PCR was used to determine BCR-ABL1(T315I) transcript levels in bone marrow of CML patients. The PCR reaction included bone marrow cDNA, specific forward primer, a reverse primer and a fluorescent TaqMan probe. The initial bone marrow cDNA sample confirmed to be T315I positive with Sanger sequencing was used as quantification

standard for follow-up samples. A standard curve was generated by diluting the cDNA from the T315I positive sample with negative control cDNA in a log-linear fashion to quantify T315I transcript levels before and after two weeks axitinib treatment. The GUS gene was used for data normalization in terms of RNA quality and cDNA synthesis. The quantification standard was measured in 4 independent runs in duplicate to estimate the between-run variation. The samples of interest (before and after axitinib treatment) were measured in three replicate analyses.

10.5 RNA sequencing

Gene expression patterns and fusion genes were identified with RNA sequencing. First, 2.5-5 µg of total RNA isolated from primary leukemic cells was treated with the Ribo-Zero™ rRNA Removal Kit (Epicentre) to remove ribosomal-DNA and RNA samples were further purified with the RNeasy Clean-up Kit (Quigen). Following sample clean up, double stranded cDNA was generated with the SuperScript™ Double-Stranded cDNA Synthesis Kit (Life Technologies). The first-strand synthesis reaction was primed with random hexamers (New England BioLabs). The RNA sequencing library was created with Nextera Technology (Epicentre), size-selected, and purified with QIAquick gel extraction Kit (Quigen). Fifty ng of cDNA was tagmented with High Molecular Weight buffer and purified with SPRI beads (Agencourt AMPure XP, Beckman Coulter). Transcriptomes were sequenced on an Illumina HiSeq2000 instrument with 100-base pairs read length. Paired-end and index sequencing were performed with Nextera and index read primers, respectively. RNA sequencing data was bioinformatically processed as previously described[254].

10.6 Fusion gene validation and analysis

In study I, fusion genes detected with RNA sequencing were further validated by capillary sequencing of cDNA. Primers were designed to amplify across fusion junctions using exon-exon fusion data from transcriptome sequencing, the NCBI nucleotide database and Primer Blast (Table 7). The amplification reaction contained double stranded cDNA, fusion gene specific primers, and Phusion or DyNazyme II DNA polymerase (Finnzymes). The resulting PCR products were size-separated on agarose gel and the band of interest purified with the NucleoSpin Gel and PCR Clean-up KIT (Macherey-Nagel). Gel-extracted cleaned up fragments were then directly sequenced or cloned into the pCRII-TOPO vector (Life Tecnologies) and the ensuing plasmid sequenced with M13 primers. The breakpoints of the fusion genes detected in patients 600 and 784 were uncovered with RNA and whole genome sequencing.

Table 7. NUP98-NSD1, MLL-ELL, ETV6-NTRK3, and STRN-ALK primer sequences

Primer use	Target	Sequence
Fusion gene validation	cNUP98-NSD1-F	AGCCTTTGGGGCCCCTGGATTTA
	cNUP98-NSD1-R	CCAAAAGCCACTTGCTTGGCTTCC
	cMLL-ELL-F	AAGTGGCTCCCCGCCCAAGT
	cMLL-ELL-R	AGGAGAACGTCCGCGCCTCT
	cETV6-NTRK3-F	CTCCCCGCCTGAAGAGCACG
	cETV6-NTRK3-R	GGCATCCAGTGACGAGGGCG
	cSTRN-ALK-F	CGGGACAGAATTGAATCAGGGA
qPCR analysis of fusion genes	gELL-MLL-F	CAGGCAGCGCTCACTCGGAAA
	gELL-MLL-R	CCTGCTTATTGACCGGAGGTGGT
	gETV6-NTRK3-F	TGGTCTGGTTCACGTTTCACTG
	gETV6-NTRK3-R	GTAAATCTTCTGCAAAAGGCAGCA
	gSTRN-ALK-F	GCTCCTATTATCCTGTCCCTTTGA
	gSTRN-ALK-R	TGGCACCATTTAGTGTCATTTAGA

11. Protein analysis

Leukemic patient cells were lysed with RIPA (study I) or SDT buffer (4% SDS, 0.1 M DTT and 0.1 M Tris; study II).

11.1 Phosphoproteomic arrays

In study I phosphoproteomic profiling of AML patient samples was done with Proteome Profiler antibody arrays (R&D Systems) according to manufacturer's instructions. About 300 µg of protein per sample was put on the arrays and the signal was visualized with fluorescently labeled streptavidin (IRDye 800 CW streptavidin; LI-COR) on an Odyssey imaging system.

11.2 Immunoblotting

In study II, immunoblotting analysis of CRKL phosphorylation status in response to axitinib treatment of patient cells derived from a CML patient carrying the T315I mutation was performed. Briefly, mononuclear cells isolated from a CML patient in lymphatic blast crisis were grown overnight in complete medium with 0.1% DMSO or increasing concentrations of axitinib (1-1,000 nM; 10-fold dilutions). The cells were then centrifuged, washed and lysed in SDT buffer (5 x 10^6 cells/condition). Protein lysates were subjected to SDS PAGE and subsequently transferred to a PVDF membrane (Immobilon). Prior to primary antibody incubation, the membranes were blocked with 5% bovine serum albumin. The following antibodies were used in the western blot analysis: rabbit anti-phospho CRKL (Cell Signaling Technologies; 3181) 1:1,000, mouse anti-alpha tubulin (Sigma Aldrich; T9026) 1:1,000, anti-mouse IRDye 680 and anti-rabbit IRDye 800 CW (LI-COR) 1:15,000. Protein bands were visualized with the LI-COR Odyssey.

11.3 Enzyme-linked immunosorbent assay (ELISA)

The effect of axitinib treatment on BCR-ABL1 autophosphorylation was examined in engineered Ba/F3 cells as previously explained with an ABL1 phosho-Tyr ELISA. Transformed Ba/F3 cells were seeded in a 96-well plate in

assay medium (RPMI-1640 with 0.1% FBS, 0.05% BSA w/v, and 1% penicillin/streptomycin) at a concentration of 40,000 cells/well and incubated for 2h at 37°C. Axitinib, ponatinib and imatinib were prepared as described in 9.5. Wells containing 100% DMSO were used as controls. Similarly as in the cell proliferation assay 25 µl of compound solution from the 5X source plate was transferred to each cell containing well following which the assay plate was incubated for additional 2 h at 37°C. The plate was then centrifuged and 80 µl supernatant was removed from each well. Cells were lysed with Cell Signaling Technologies lysis buffer (9803; 100 µl/well) containing 1% SDS, protease (Sigma P8340) and phosphatase (Sigma P0044 and P5726) inhibitors. The assay plate was then put on a shaker for 10 minutes at 4°C. Subsequently, 100 µl of lysate per well was transferred to a goat anti-rabbit 96-well coated ELISA plate (Pierce 15135) previously incubated with rabbit anti-ABL1 antibody (Cell Signaling Technologies 2862). The ELISA plate was left to stand at room temperature for 1 h and then washed 4 times with ELISA wash buffer (Cell Signaling Technologies; from kit 7903). The secondary antibody used was mouse monoclonal (IgG2b) anti-phospho-Tyr antibody (Santa Cruz Biotechnology SC508 HRP; 1:5,000). The signal detection was done with TMB substrate (Santa Cruz Biotechnology; SC286967) and absorbance was measured at 655 nm during color development or at 450 nm after reaction stoppage with 0.16 M sulphuric acid stop solution.

11.4 Biochemical kinase assays

In study II, to evaluate whether axitinib inhibits the kinase activity of ABL1 and ABL1(T315I) a microfluidic mobility shift assay was performed. The reaction volume was 50 µl and consisted of 1 nM GST-tagged intracellular kinase domain of human recombinant ABL1 or ABL1(T315I), 3 µM phosphoacceptor peptide (5'FAM-EAIYAAPFAKKK-OH; Caliper Life Sciences), axitinib (11-dose 3-fold serial dilutions) or DMSO only, 1 mM DTT, 0.002% Tween-20 and 25 mM HEPES (pH 7.1) containing 5mM $MgCl_2$. The assay was started by adding ATP (5 µM final concentration) and reactions were incubated for 20 minutes and then for additional 90 minutes at room temperature. The reactions were terminated with addition of EDTA (pH 8). The extent of electrophoretic separation of the fluorescently labeled peptide substrate and phosphorylated product was detected with the LabChip EZ Reader II (Caliper Life Sciences). Inhibitory constants (K_i) were generated with the Morrison equation[255] for tight-binding competitive inhibition utilizing the non-linear regression method, best fit enzyme concentration value and experimentally obtained ATP Km (~ 4 µM).

11.5 Recombinant ABL1 production

A construct containing residues 229-515 of human ABL1a (NM_005157.4) previously subcloned in an insect cell expression vector was acquired from GenScript. The T315I mutation was introduced in this construct with site-directed mutagenesis. Recombinant baculoviruses, produced with the Bac-to-Bac method (Invitrogen), were exploited to infect Sf21 insect cells at 27°C. In addition, co-infection of ABL1 viruses with baculoviruses expressing human YopH tyrosine phosphates at *moi* = 0.01 was performed to obtain non-

phosphorylated protein variants. Infected insect cells were isolated after 72 h and stored at -80°C before purification. Cell pellets were then lysed with lysis buffer and mixed at 4°C for 1 h. The cell lysate was then centrifuged at 5,000 x *g* for 1 h after which the supernatant was incubated for 3 h at 4°C with ProBond resin (Invitrogen). The ProBond resin bound ABL1 was washed, eluted on an Econo column (Bio-Rad), and treated with TEV protease during overnight dialysis against wash buffer. The material obtained from the dialysis step was put on a fresh column of ProBond resin, which was beforehand equilibrated with post-dialysis buffer, allowing for collection of the flow-through that contained the detagged ABL1(T315I). The flow-through was then dialyzed against delivery buffer. The same procedure was applied for the isolation of the kinase domain of ABL1, except that $MgCl_2$ and ADP-NaOH were excluded from all buffers. The extracted proteins were snap-frozen in liquid nitrogen and stored at -80°C. Protein concentrations were measured with Coomassie Plus Protein reagent (Pierce). Buffers used for recombinant protein productions are depicted in Table 8.

Table 8. Buffers used for the extraction of recombinant kinase domains of ABL1 and ABL1(T315I)

Buffer	Contents
Lysis buffer	50 mM Tris-HCl pH 8 200 mM NaCL 10 mM $MgCl_2$ 5 mM ADP-NaOH pH 7.5 0.25 mM TCEP 2 µM leupeptin 1 tablet EDTA free protease inhibitor
Wash buffer	50 mM Tris-HCL pH 8 400 mM NaCl 20 mM imidazole-HCL pH 8 10 mM $MgCl_2$ 2.5 mM ADP-NaOH pH 7.5 0.25 mM TCEP 1 µl leupeptin
Elution buffer	50 mM Tris-HCL pH 8 400 mM NaCl 250 mM imidazole-HCL pH 8 10 mM $MgCl_2$ 2.5 mM ADP-NaOH pH 7.5 0.25 mM TCEP 1 µl leupeptin
Delivery buffer	25 mM HEPES-NaOH pH7.2 250 mM NaCl 5 mM $MgCl_2$ 2.5 mM ADP-NaOH pH 7.5 20% (v/v) glycerol 0.25 mM TCEP

11.6 X-ray crystallography

Once thawed, ABL1 and ABL1(T315I) proteins were diluted with cold 25 mM Tris-HCl (pH 8), 150 mM NaCl, and 5 mM DTT to a concentration of 10 µM

(~0.3 mg/ml). Then equal parts of 20 µM axitinib (diluted in the same buffer) were added to the protein solution and the mixture was incubated on ice for 1 h. Millipore centrifugal concentrator was used to concentrate the protein-axitinib solution to ~20 mg/ml. A Mosquito liquid handling device was used to generate crystals in SBS format MRC2 crystallization plates via sitting drop vapor diffusion. A mixture of 196 nl protein-axitinib solution (17 mg/ml) and 211 nl reservoir solution (15% w/v PEG 3350, 10 mM $MgCl_2$, 5 mM $NiCl_2$, 5% v/v glycerol, and 100 mM HEPES) at 13°C led to the formation of ABL1(T315I) crystals. On the other hand, ABL1 crystals were obtained from a mixture of 150 nl protein-axitinib solution (18 mg/ml) and 190 nl reservoir solution (0.1 M NH_4Cl, 20% w/v PEG 3350 and 5% v/v ethylene glycol). The crystal drops were first cryo-protected with 4 µl reservoir solution (20% glycerol for ABL1(T315I) and 20% ethylene glycol for ABL1) and then harvested straight from the crystallization drop and snap-froze in liquid nitrogen. The Advanced Photon Source synchrotron (Argonne National Laboratories) was used to collect the X-ray data sets at beamline 17-ID, wavelength 1.00 Å, and temperature 100 K. For the axitinib:ABL1(T315I) complex the R_{merge} was 0.055 for data ranging between 65.58 and 2.40 Å, whereas for the axitinib:ABL1 complex R_{merge} was 0.057 for data in the range of 111.81-2-20 Å. Solving the structures involved molecular replacement in PHASER[256] taking the refined ABL1(T315I) atomic coordinates (axitinib:ABL1) or ABL1 structure (wwPDB ID 3IK3; axitinib:ABL1(T315I)) as a starting point, repetitious model building and refinement with Coot[257] and REFMAC5[258], and final refinement and validation with the PHENIX system[259]. For both crystal structures, Ramachandran plots showed that majority of the residues are in favored regions (97.9% (ABL1(T315I) and 97.1% (ABL1)). The structural data has been deposited in Protein Data Bank under accession numbers 4TWP (axitinib:ABL1(T315I)) and 4WA9 (axitinib:ABL1).

12. Statistics

Statistical analysis was performed with GraphPad Prism software (studies I and II) and R interface (SA I). Drug response profile correlations were evaluated with Pearson or Spearman correlation test, whereas two-tailed Student *t* test was used to determine correlation between mutational profiles and drug sensitivity. Skewness of drug sensitivity distribution was assessed with one-sided D'Agostino test in the R-package 'moments' v. 0.13. Difference in sensitivity between BRAF(V600E) or RAS mutated and wild-type cells to BRAF and MEK inhibitors, respectively, was detected with Wilcoxon rank-sum test. ROC curve analysis was applied to estimate the predictive accuracy of the different drug sensitivity scoring metrics in differentiating active drugs from inactive ones using R-packages 'verification' and 'pROC'. Adjusted Rand, Jaccard and Fowlkes-Mallows indices were used to measure similarities in drug clustering in terms of mode of action between DSS, Activity Areal and pIC_{50}. The difference in T315I transcript levels in bone marrow before and after axitinib treatment was inferred with un-paired two-tailed *t* test with Welch's correction. A *P*-value ≤ 0.05 was considered statistically significant.

RESULTS

13. Development of an Individualized Systems Medicine platform

To identify and optimize novel treatment strategies for leukemia patients an Individualized Systems Medicine (ISM) platform was developed (Figure 12). The ISM platform encompasses comprehensive functional and molecular profiling of leukemia patient cells with an oncology drug collection and genomic and transcriptomic sequencing, respectively. This approach facilitates evaluation of *ex vivo* drug sensitivity of cancer cells and uncovers underlying disease mechanisms and molecular drivers. In addition, it holds promise for rapid translation of results and identification of drugs that can be repurposed for treatment of leukemia patients. Furthermore, this strategy makes it possible to monitor disease progression, therapy response and clonal evolution by studying consecutive samples from the same patient before and after relapse. The functional and molecular information is also integrated with clinical features to guide therapy decisions.

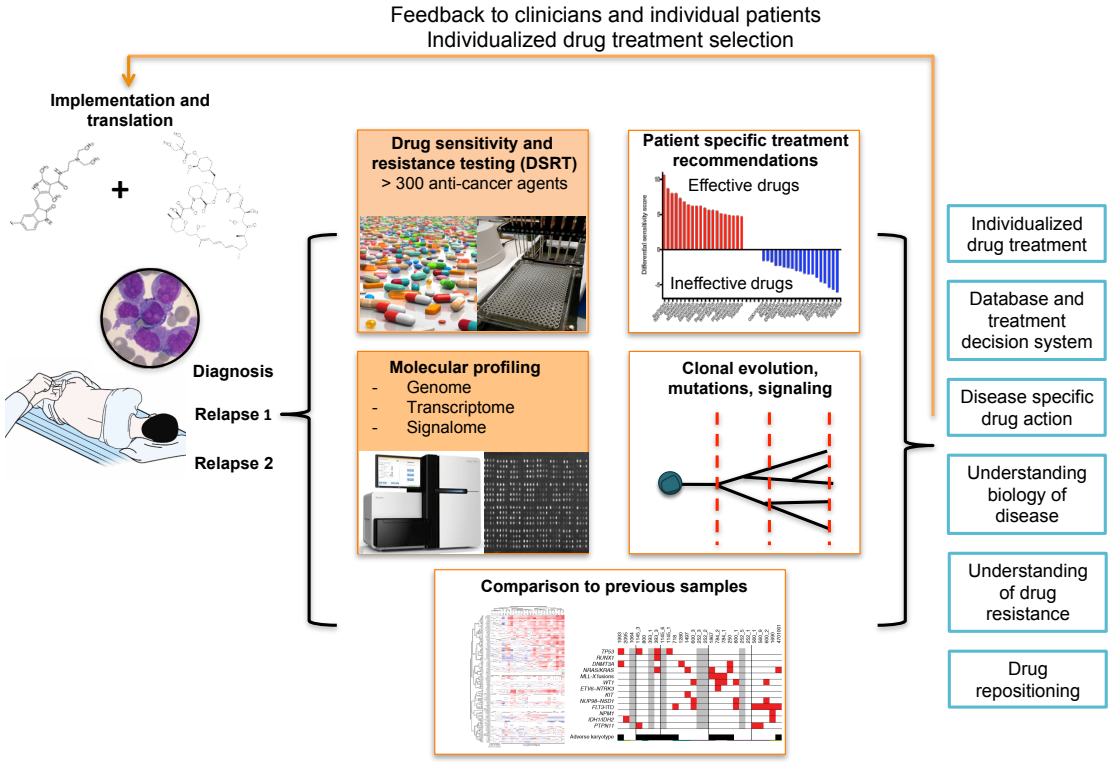


Figure 12. Integrated individualized systems medicine platform involving ex vivo drug sensitivity and resistance testing (DSRT) and deep molecular profiling as means of personalized therapy optimization for cancer patients.

13.1 DSRT of leukemia patient cells

To establish an unbiased functional readout for cellular vulnerabilities of individual cancer samples, an *ex vivo* drug sensitivity and resistance testing (DSRT) screening platform was developed that covers the approved cancer small molecule pharmacopeia and the active substances of emerging investigational and experimental cancer drugs. The availability of a large

number of drugs that target oncogenic signals and signaling pathways allows for an increasingly comprehensive functional profiling of cancer patient cells and their dependencies and addictions to cellular signals. This thesis describes the analysis of *ex vivo* drug sensitivity profiles of 28 primarily relapsed and refractory AML (study I and SA I), 6 Ph+ (study II), and 7 healthy donor bone marrow samples (studies I-II, and SA I). With the established DSRT culture conditions, majority of patient and healthy donor control samples exhibited relative cell viability above 50% following 72 h incubation without drug treatment (Figure 13). The malignant cell count of each sample did not correlate with the relative cell viability after 72 h incubation (Figure 12). Moreover, drug sensitivity data analysis divulged that the DSRT platform is robust and reproducible and that the profiles of healthy bone marrow control samples were highly in accordance (Figure 14).

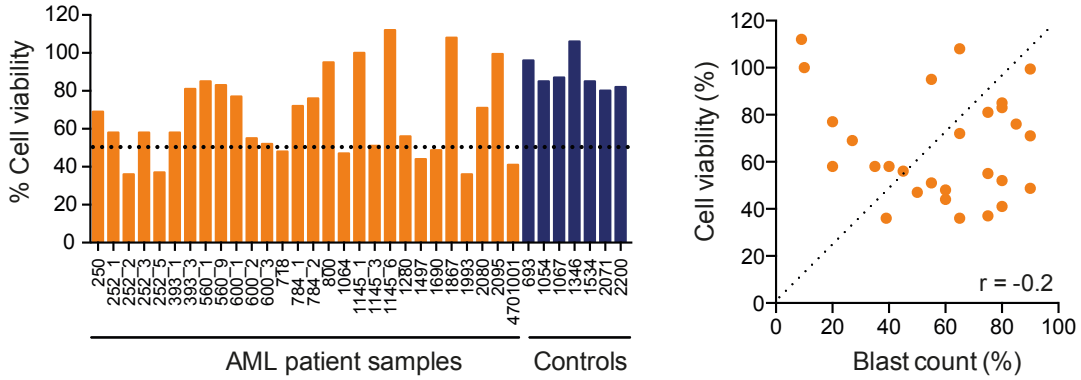


Figure 13. Relative ex vivo cell viability in AML patient samples after a 72 h incubation period in 384-well plates without drug treatment (left) and correlation with blast count (right).

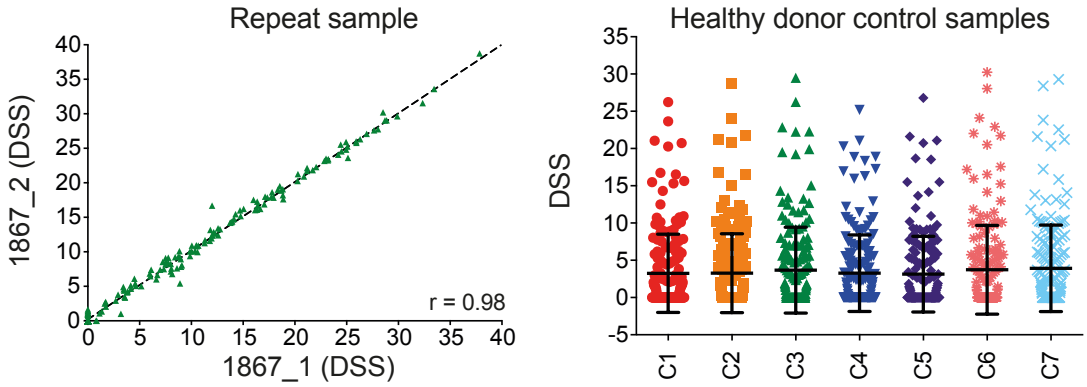


Figure 14. DSRT data from sample replicates (left) and from different healthy donor control samples (right) exhibit excellent correlation. DSS correlation plot of a patient sample screened twice (as two independent experiments) and DSS scatterplots of each of the 7 healthy donor samples with mean and standard deviation as error bars.

13.2 DSRT profiles of AML patient samples

Numerous targeted signal transduction inhibitors exhibited little or no effect in the controls and in majority of AML patient samples and only a subset of patient samples display selective responses to these agents, illustrating that the inhibitors are hitting subgroup specific addictions. Moreover, drug

sensitivity profiles of AML patient samples were distinctly different from those of control samples, suggesting that leukemia specific inhibitory effects are detected with the DSRT screening platform (Figure 15A). The differences between healthy donor and leukemia patient samples could mostly be attributed to targeted drugs (Figure 15A-B), as the overall sensitivity to classical chemotherapeutics was largely comparable in patient and control samples with the exception of cytarabine (Figure 15A,C). Hence, chemotherapy agents have a narrower therapeutic window and their efficacy *in vivo* is more challenging to predict based on *ex vivo* drug sensitivity data. Meaningful leukemia-selective responses were detected to the broad TKIs dasatinib (36% of the samples) and sunitinib (36%), MEK inhibitors (trametinib 36%), rapalogs (temsirolimus 32%), and other TKIs such as foretinib (32%), ponatinib (25%), ruxolitinib (25%), dactolisib (25%), MK-2206 (21%), sorafenib (21%), and quizartinib (18%; Figure 15D-E).

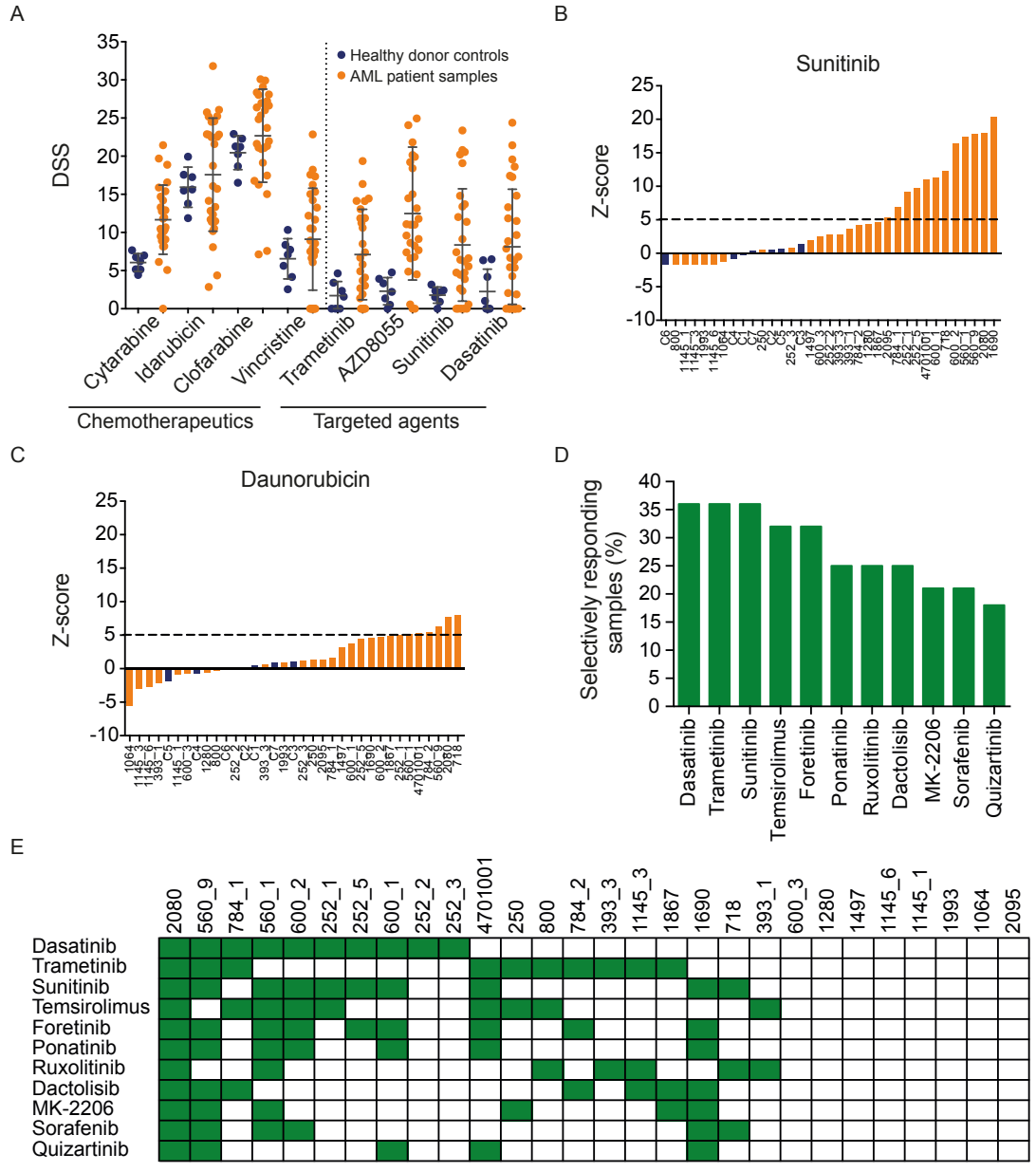


Figure 15. Targeted agents display leukemia-selective responses in AML. A. Comparative analysis of ex vivo drug responses (DSS) to chemotherapeutics (left) and targeted agents (right) in 28 AML and 7 control samples. Error bars signify mean with standard deviation. B-C. Distribution of sensitivity to sunitinib (broad TKI) and daunorubicin (topoisomerase II inhibitor) in the study cohort expressed as standard deviations from the mean control DSS response (Z-score). D-E. The percentage of patient samples responding to select targeted agents as assessed by sDSS represented with a bar graph and a heatmap of relationships between different selective drug responses.

In order to measure differential drug effect as compared to healthy donor control samples, unsupervised hierarchical complete linkage clustering with spearman correlation distance measure of sDSS was employed. This approach facilitated stratification of patients based on drug efficacy patterns across all drugs screened as well as taxonomy of drugs in terms of responses among all patients in this particular disease setting. The cluster analysis showed that each AML patient sample has an overall distinct drug sensitivity profile. However, based on correlative drug responses the AML patient samples could be functionally classified in 5 robust subgroups. All of the patient sample subgroups could be characterized by either sensitivity or not sensitivity to certain drug classes (Figure 16). Majority of patient samples exhibited selective responses to navitoclax (BCL-2/BCL-XL inhibitor), HSP90 and histone deacetylase (HDAC) inhibitors. Subgroup I was largely insensitive to the tested compound panel with the exception of navitoclax. Subgroup II was defined by selective sensitivity to immunomodulators (dexamethasone and prednisolone), MEK and Janus-activated kinase (JAK) inhibitors. Contrary to subgroups I and II, subgroups III, IV and V displayed increased sensitivity to a wide range of TKIs, suggesting that their AML was either driven or addicted to RTK signaling pathways. In addition to TKIs, subgroup III showed sensitivity to HSP90, HDAC and PI3K/mTOR inhibitors similarly to samples falling in subgroups IV and V but with lower intensity. Subgroup IV was also defined by strong sensitivity to MEK and PI3K/mTOR inhibitors, whereas subgroup V by potent selective responses to RTK inhibitors (targeting ABL1, VEGFR, PDGFR, FLT3, and KIT), PI3K/mTOR and topoisomerase II inhibitors. Strikingly, almost 70% of the AML patient samples exhibited moderate to strong sensitivity to TKIs in line with previous studies[235].

Clustering of compounds across AML and control samples revealed that inhibitors with related mode of actions in majority of cases highly correlated (clustered together) such as immunomodulators, MEK inhibitors, HSP90 inhibitors, HDAC inhibitors, PI3K/AKT/mTOR inhibitors, ABL1, KIT and VEGFR targeting TKIs, and topoisomerase II inhibitors. These findings are not only supporting the notion that anti-leukemia specific drug responses are detected with the DSRT platform, but also that the DSRT analysis is consistent and reproducible and can derive biologically and clinically relevant data. However, unexpected clusters were also observed with quizartinib's responses correlating with those of topoisomerase II inhibitors, whereas ponatinib's clustered with cytarabine, HSP90 and HDAC inhibitors. These revelations suggest that the activity of these agents in AML may be functionally linked and could aid in elucidating critical molecular mechanisms of action in this disease setting. Moreover, AML specific drug clustering

results can be utilized to design subtype-specific drug combinations strategies to be clinically explored, which would not be otherwise identified without unbiased DSRT profiles. For example, responses of MEK inhibitors highly correlate with either JAK or PI3K/mTOR inhibitors in subgroups II and IV, respectively. In addition, FLT3 inhibition is associated with dasatinib (does not target FLT3) sensitivity in subgroup V, especially in M5 categorized patients (Figure 17). This indicates that these compounds may inhibit the same or linked signaling pathways and poses the question whether a combined treatment will be synergistic in that particular patient population.

13.3 Associations between functional and molecular profiles

To determine whether there are links between genomic and drug sensitivity data, the distribution of mutations in most frequently altered genes in AML, as per TCGA[76] data, with DSRT-based sample taxonomy was assessed. An enrichment of *FLT3*-ITD mutations in samples falling in subgroup V was observed, which was the most tyrosine kinase-addicted group. Hence, *FLT3*-ITD mutational status can serve as an indicator whether a patient would respond to tyrosine kinase inhibition. Furthermore, subgroup V was defined by sensitivity to a number of FLT3 inhibitors such as foretinib, quizartinib and sunitinib, indicating that sensitivity to FLT3 inhibitors link to activating *FLT3* mutations. However, 4 out of 5 patient samples in this subgroup exhibited strong sensitivity to dasatinib that does not have FLT3 inhibitory activity, implying that AMLs with *FLT3* mutations are also reliant on additional tyrosine kinase signals. Overall, dasatinib sensitivity was highest in patient samples harboring *FLT3* mutations (P = 0.0034) and AML M5-diagnosed samples (P = 0.0004*)* (Figure 17). In particular, FLT3, ATP-competitive mTOR, topoisomerase II inhibitors and navitoclax were selective in AML-M1 samples, whereas navitoclax, mTOR inhibitors and the IGF1R/InsR inhibitor linsitinib exhibited selective responses in AML-M2 patient samples. Besides selective responses to dasatinib, AML M5 samples displayed enhanced sensitivity to tivozanib, a number of broad TKI targeting FLT3, HSP90 inhibitors, mTOR inhibitors and the MEK inhibitor refametinib (Figure 17). Therefore, the FAB classification of AML cases may serve as a guideline for some types of personalized therapies.

Activating mutations in *RAS* and MLL fusions were associated with MEK inhibitor sensitivity, whereas TP53 mutations were enriched in subgroups I and II and linked to adverse karyotype. While all *RAS* mutated samples exhibited MEK inhibitor sensitivity, not all MEK inhibitor sensitive samples harbored *RAS* mutations. This indicates that there are additional factors that confer MEK inhibitor sensitivity in AML. Associations between mutations and/or fusions and epigenetic modulation in subgroups III and IV were also observed. Hence, some links from phenotype to genotype were detected, but for most drug responses detected no clear link to genotype could be elucidated.

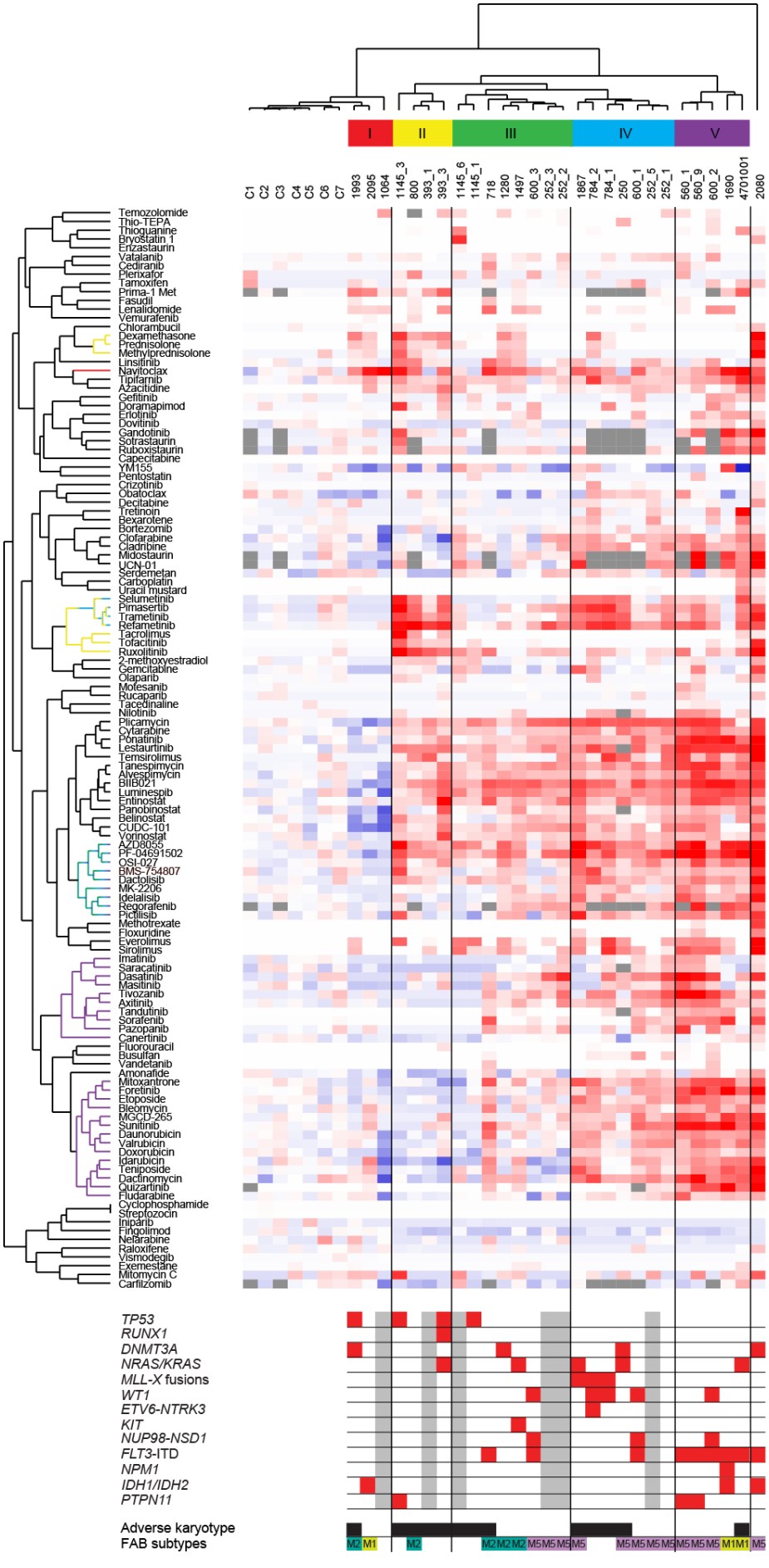

I
II
III
IV
V
C1 C2 C3 C4 C5 C6 C7 1993 2095 1064 1145_3 800 393_1 393_3 1145_6 1145_1 718 1280 1497 600_3 252_3 252_2 1867 784_2 784_1 250 600_1 252_5 252_1 560_1 560_9 600_2 1690 4701001 2080
Temozolomide
Thio-TEPA
Thioguanine
Bryostatin 1
Enzastaurin
Vatalanib
Cediranib
Plerixafor
Tamoxifen
Prima-1 Met
Fasudil
Lenalidomide
Vemurafenib
Chlorambucil
Dexamethasone
Prednisolone
Methylprednisolone
Linsitinib
Navitoclax
Tipifarnib
Azacitidine
Gefitinib
Doramapimod
Erlotinib
Dovitinib
Gandotinib
Sotrastaurin
Ruboxistaurin
Capecitabine
YM155
Pentostatin
Crizotinib
Obatoclax
Decitabine
Tretinoin
Bexarotene
Bortezomib
Clofarabine
Cladribine
Midostaurin
UCN-01
Serdemetan
Carboplatin
Uracil mustard
Selumetinib
Pimasertib
Trametinib
Refametinib
Tacrolimus
Tofacitinib
Ruxolitinib
2-methoxyestradiol
Gemcitabine
Olaparib
Motesanib
Rucaparib
Tacedinaline
Nilotinib
Plicamycin
Cytarabine
Ponatinib
Lestaurtinib
Temsirolimus
Tanespimycin
Alvespimycin
BIIB021
Luminespib
Entinostat
Panobinostat
Belinostat
CUDC-101
Vorinostat
AZD8055
PF-04691502
OSI-027
BMS-754807
Dactolisib
MK-2206
Idelalisib
Regorafenib
Pictilisib
Methotrexate
Floxuridine
Everolimus
Sirolimus
Imatinib
Saracatinib
Dasatinib
Masitinib
Tivozanib
Axitinib
Tandutinib
Sorafenib
Pazopanib
Canertinib
Fluorouracil
Busulfan
Vandetanib
Amonafide
Mitoxantrone
Foretinib
Etoposide
Bleomycin
MGCD-265
Sunitinib
Daunorubicin
Valrubicin
Doxorubicin
Idarubicin
Teniposide
Dactinomycin
Quizartinib
Fludarabine
Cyclophosphamide
Streptozocin
Iniparib
Pirfenidone
Nelarabine
Raloxifene
Vismodegib
Exemestane
Mitomycin C
Carfilzomib
TP53
RUNX1
DNMT3A
NRAS/KRAS
MLL-X fusions
WT1
ETV6-NTRK3
KIT
NUP98-NSD1
FLT3-ITD
NPM1
IDH1/IDH2
PTPN11
Adverse karyotype
FAB subtypes
M2 M1 M2 M2 M2 M2 M5 M5 M5 M5 M5 M5 M5 M5 M5 M5 M5 M1 M1 M5

Figure 16. Ex vivo anti-cancer sensitivity testing identifies promising therapies for subgroups of AML patients. A heatmap of drug sensitivity profiles (expressed as sDSS) of 28 AML and 7 control samples separates AML patient samples in 5 functional taxonomic groups. Selective drug responses are depicted in red, whereas drugs that exhibit lower efficacy in AML patient samples than control samples are in blue. The unsupervised clustering was performed with the complete linkage method with Euclidian (samples) and Spearman (drugs) distance measures. Significant mutations and gene fusions are also depicted to correlate drug sensitivity and mutation data.

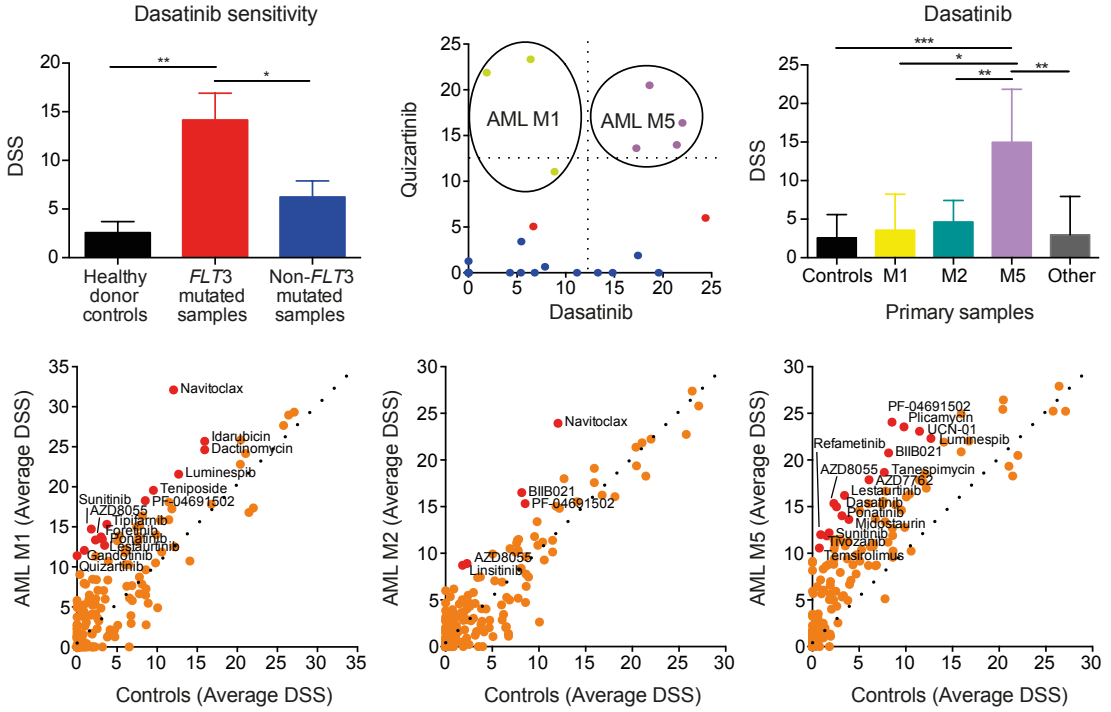


Figure 17. Sensitivity to dasatinib in AML patient and healthy donor control samples where the AML samples are stratified based on FLT3 mutational status or FAB subtype. Correlations between drug sensitivity profiles of AML M1, M2 or M5 and healthy bone marrow controls further highlight compounds (in red) with cancer-specific activity in the AML subgroups.

13.4 A subset of AML patient samples is highly sensitive to MEK inhibitors

MEK inhibitors have been explored in clinical trials for AML in the past without significant success, suggesting that either MEK is not a relevant target in the AML setting or more likely that the appropriate subgroup of patients has not been identified. The four MEK inhibitors included in the oncology collection in study I, selumetinib, trametinib, refametinib and pimasertib, exhibited highly consistent response pattern across the AML patient samples and no or very minor effect in the healthy donor control samples (Figure 18). The overall drug response pattern to MEK inhibitors varied in different samples, with 12 samples displaying no, 7 partial and 9 significant sensitivity. Together this data indicates that the DSRT approach can stratify patient samples in terms of MEK inhibitor sensitivity and identify candidate patients for tailored MEK inhibitor therapy. No clear link between MEK signaling and a specific RTK was observed, as no RTK inhibitor exhibited analogous sensitivity pattern with

the MEK inhibitors. This suggests that the mechanism underlying MEK addiction may be dependent on other factors such as DUSP6 (phosphatase specifically inactivating pERK2) expression, which has been shown to be an independent predictor of MEK inhibitor sensitivity[260].

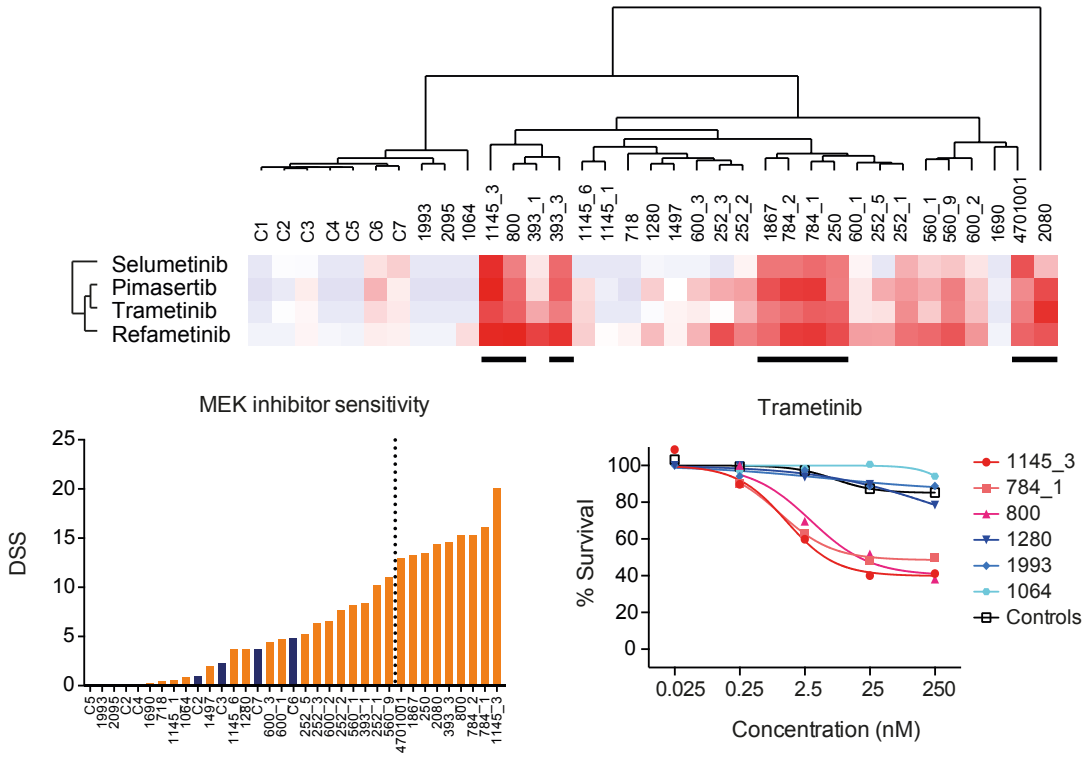


Figure 18. MEK inhibition is a potentially promising therapeutic strategy in a subset of AML patients. The 9 samples underlined in the top panel are separated by the dashed line in the bottom left panel. Dose response curves for trametinib treatment in 3 selective (red), 3 insensitive (blue) and average control (n = 7; black) samples show a highly cancer cell–selective response pattern with a low level of activity in control samples, suggesting a potential for wide therapeutic window.

13.5 mTOR and upstream signals as molecular targets in AML

Ten out of the 28 AML patient samples were sensitive to rapamycin and its analogs (temsirolimus and everolimus) (Figure 19), a class of indirect mTOR complex 1 inhibitors that are approved for oncology and non-oncology indications. Drug sensitivities to the 3 rapalogs were highly concordant among the samples with everolimus and sirolimus clustering together in the full DSRT dataset (Figure 16). Of the three, temsirolimus consistently reduced viability of AML cells with highest intensity. While everolimus and sirolimus clustered relatively close to ATP-competitive PI3K/AKT/mTOR pathway inhibitors, they grouped closest to methotrexate and floxuridine and temsirolimus with lestaurtinib, ponatinib and cytarabine. This suggests that rapalogs and the ATP-competitive PI3K/AKT/mTOR inhibitors, at least in part, affected different cellular mechanisms. One striking difference between the two compound types was that while rapalogs exhibited extremely wide therapeutic windows with effects on sensitive patient samples often in the sub-nanomolar range and no toxicity in the control cells until low micro-molar concentrations, all the ATP-competitive PI3K/AKT/mTOR pathway inhibitors had considerable

effects on the control cells already at concentrations relatively close to the cancer-specific effective concentrations. One patient sample (600_2) out of 7 that were significantly sensitive to rapalogs did not respond selectively to an ATP-competitive mTOR inhibitor (AZD8055, targeting mTORC1 and mTORC2), a pan PI3K inhibitor (pictilisib) or an AKT inhibitor (MK-2206). Similarly, several (1280 and 1497) of the samples that selectively responded to PI3K/AKT/mTOR inhibitors did not respond strongly to rapalogs (Figure 19). Therefore, while rapalogs emerge as promising agents in a subset of AML samples that could be identified through DSRT, the mechanism of sensitivity to rapalogs appears to be more complex that just through an activation of the PI3K/AKT/mTOR pathway.

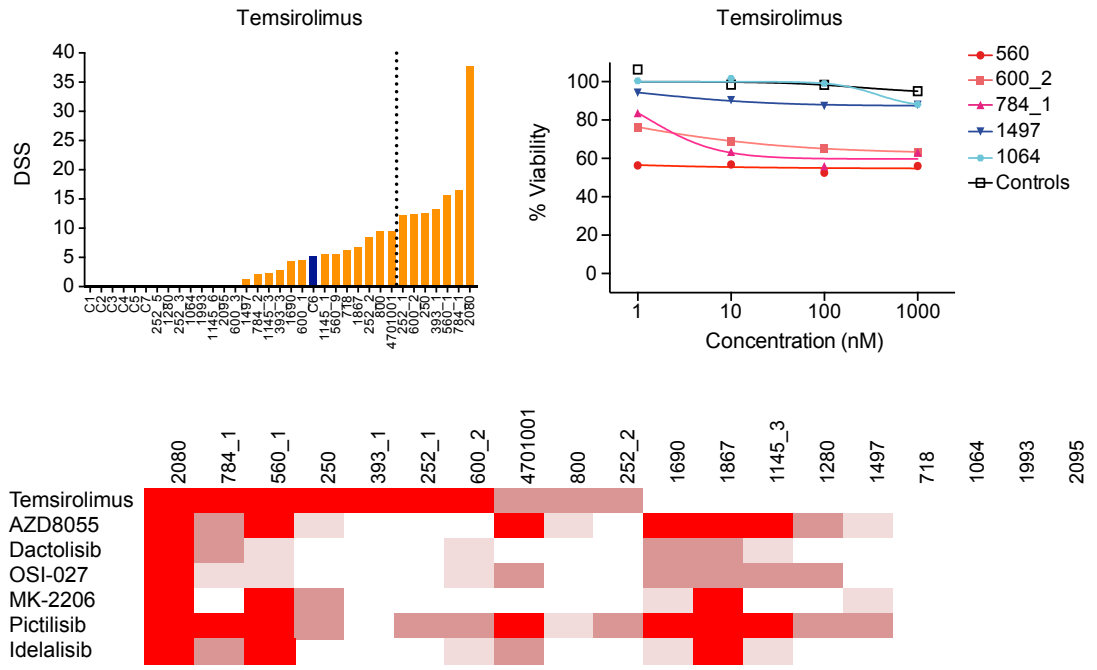


Figure 19. Rapalogs induce selective ex-vivo responses in a subset of AML patient samples and their activity profile is distinct from other PI3K/AKT/mTOR inhibitors. Distribution of temsirolimus sensitivity in the AML and control samples highlighted 7 samples with significant responses (separated with a dashed line). Temsirolimus dose response curves for 3 responders, 2 non-responders and the average of responses in controls samples are shown shown in red, blue and black, respectively. Heatmap depicting the inter-relationships of samples responding to temsirolimus, ATP-competitive mTOR inhibitors (AZD8055, dactolisib, and OSI-027), AKT inhibitor (MK-2206), and PI3K inhibitors (pictilisib and idelalisib. Responses are depicted as strong (red), intermediate (pink) or low (pale pink).

13.6 Drug sensitivity profiles of Ph+ leukemia patient samples

In addition to AML, the DSRT platform was also applied to 3 CML and 3 Ph+ ALL patient samples (study II). Four samples from the Ph+ patient cohort harbored the BCR-ABL1(T315I) mutation (155, 542, 1278 and 1408), one had no detectable point mutations (410) and one of the CML patient samples carried BCR-ABL1(E255K) and BCR-ABL1(V299L) mutations (651). Patient samples with T315I mutations were not sensitive to imatinib, dasatinib and nilotinib, but exhibited sensitivity to ponatinib as expected (Figure 20). Varied

degree of sensitivity to a number of inhibitors targeting main BCR-ABL1 downstream effector signals were observed such as BCL-2/BCL-XL, MEK, JAK, and PI3K/AKT/mTOR. Furthermore, immunomodulators and inhibitors of KIT, PDGFR and VEGFR showed activity in Ph+ patient cells (Figure 20). In addition to ponatinib, patient samples carrying the T315I mutation displayed potent selective responses to the aurora kinase inhibitor danusertib, a probe with known efficacy against T315I-positive cells[261], PI3K/mTOR inhibitors and unexpectedly to the VEGFR2 inhibitor axitinib. The median IC_{50} values detected for axitinib were 20-fold lower in patient cells harboring T315I mutations in comparison to T315I-negative Ph+ cells. Interestingly, no other VEGFR, PDGFR or KIT targeting TKI showed comparable efficacy in the patient samples with T315I mutations, indicating that axitinib is inhibiting a different target in these cells. Axitinib, highly-selective for VEGFR[262], was developed by Pfizer and is currently approved for refractory renal cell carcinoma patients as an angiogenesis inhibitor[263]. Axitinib has not been previously explored in the context of biochemical or cellular BCR-ABL1 inhibition.

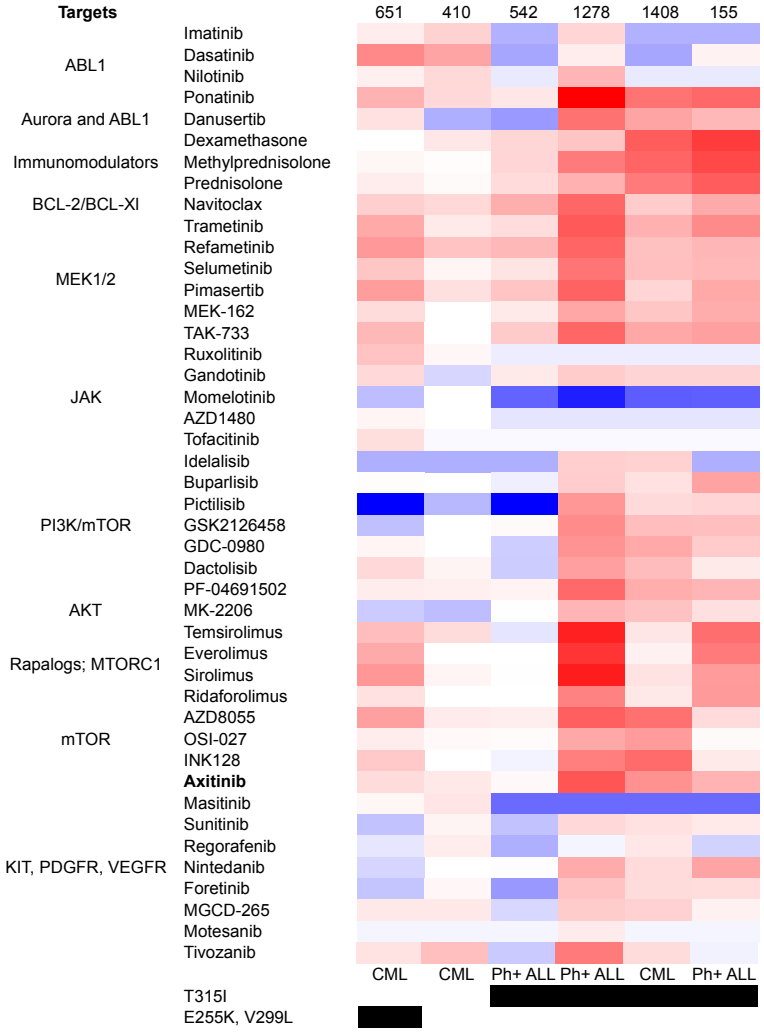


Figure 20. Heatmap of selected drug sensitivities and their main targets in CML and Ph+ ALL patient samples. BCR-ABL1 mutational status is also shown to correlate genotype to phenotype.

13.7 Drug sensitivity studies in cell lines

To assess the workability of the DSS in more controlled settings, the DSS analysis pipeline was applied to drug sensitivity data from the CCLE study. In particular, the sensitivity of a MEK inhibitor (selumetinib) in wild-type or *RAS* mutated hematopoietic and lymphoid cell lines was explored (Figure 21). Comparison of the DSS with AA metrics (used in the CCLE study) or conventional IC_{50} analysis illustrated that DSS and AA delivered comparable power in detecting selective responses of the MEK inhibitor in *RAS* mutated cell lines (P = 0.0003 and P = 0.0032) in comparison to the wild-type counterpart, whereas the IC_{50} metric did not show statistically significant difference in MEK inhibitor sensitivity (P = 0.0954). In addition, the DSS was capable of further distinguishing the difference in sensitivity between wild-type and mutated cell lines, as the DSS scored the drug responses in wild-type cells lower than the Activity Area. These data show that drug response quantification with DSS surpasses conventional drug sensitivity scoring metrics such as IC_{50} and has similar selectivity to the Activity Area.

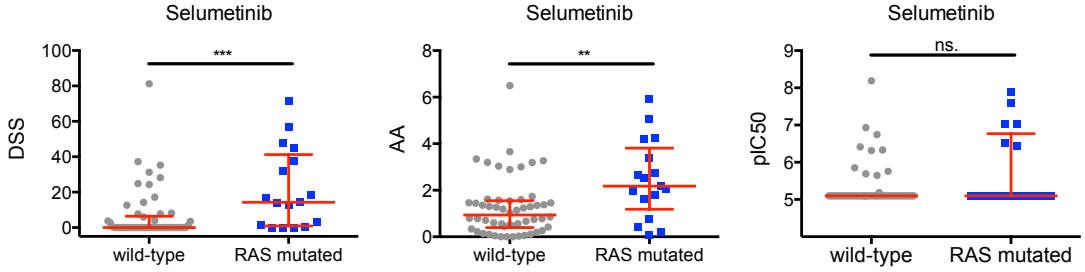


Figure 21. Distribution of sensitivities to selumetinib in hematopoietic and lymphoid cell lines quantified with DSS, Activity Area (AA), and pIC_{50}. Ns-non significant, ***-P < 0.0005, **-P < 0.005.

In order to determine whether the observed activity of axitinib in Ph+ patient cells with T315I mutations was as a result of specific inhibition of BCR-ABL1(T315I), murine pro-B Ba/F3 cells transformed to stably express BCR-ABL1 or BCR-ABL1(T315I) were utilized. In this cell model the ability of axitinib to inhibit ABL1 autophosphorylation and BCR-ABL1-dependent cell proliferation was evaluated. In line with the effects of ponatinib, axitinib potently and selectively diminished autophosphorylation of ABL1(T315I) and inhibited the growth of BCR-ABL1(T315I) positive Ba/F3 cells in a dose dependent manner (Figure 22). In contrast, the effects of axitinib in engineered Ba/F3 cells expressing BCR-ABL1 were 10-fold lower. In a larger panel of transformed Ba/F3 cells with clinically important BCR-ABL1 resistance mutations, axitinib exhibited highly selective efficacy towards cells harboring gatekeeper mutations. Thus, axitinib can specifically and efficiently target gatekeeper-mutant BCR-ABL1.

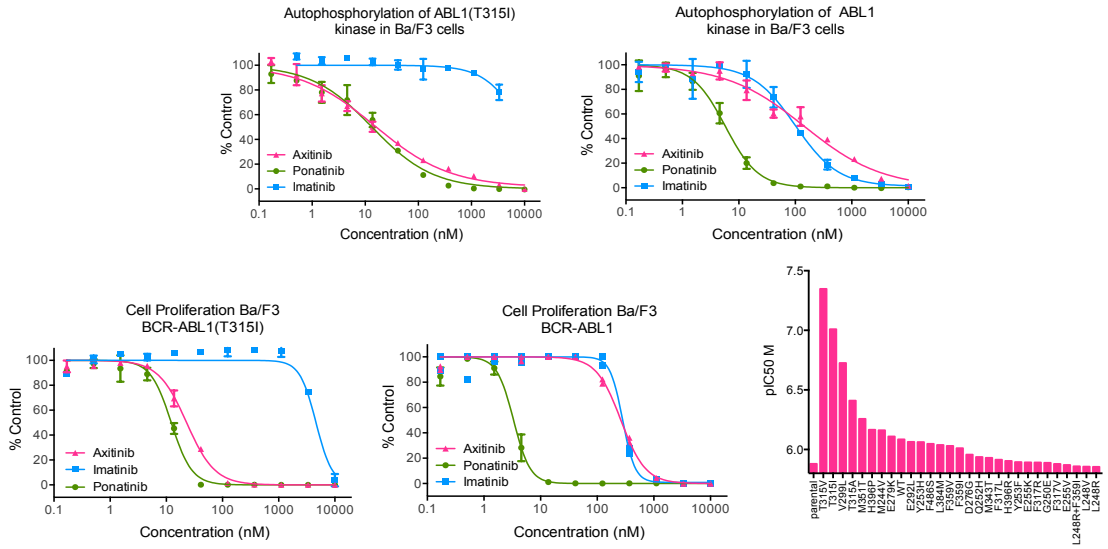


Figure 22. Axitinib blocks BCR-ABL1(T315I) autophosphorylation and proliferation of Ba/F3 cells expressing BCR-ABL1(T315I) in a dose dependent manner as measured with phospho ABL1 ELISA and resazurin cell viability assay, respectively. Error bars indicate standard deviation of mean of several repeated experiments.

14. Clinical implementation of DSRT data

Several criteria had to be met for DSRT data to be considered translatable, namely that distinctive leukemia selective response was detected, the translation candidate drug/s were accessible for compassionate or off-label use, and for the patient in question no other treatment strategy to be feasible. In the studies included in this thesis, DSRT-guided treatment was given to 8 AML and 1 CML patient. Responses to clinical therapy were evaluated according to ELN criteria[9] and 3 out of 9 patients achieved a response to DSRT-optimized therapy (Table 9). One patient (600; full disease course in section below) achieved complete remission with incomplete platelet recovery on dasatinib-sunitinib-temsirolimus treatment and two patients (718 and 800) achieved morphologic leukemia-free state on sorafenib-clofarabine and dasatinib-clofarabine-vinblastine, respectively.

In addition, responses, not meeting ELN response criteria were also detected in 4 other AML patients and 1 CML patient. Five days of dasatinib-sunitinib treatment led to rapid clearance of peripheral blood blasts in patient 560, but treatment had to be terminated due to gastrointestinal toxicity. Patient 252 (AML with 3 previous relapses) attained 8-week progression free survival on dasatinib treatment. In patient 784 the combination of dasatinib, sunitinib and temsirolimus caused a reduction in bone marrow blasts (from 70% to 35%), but the response was short-lived due to selection of a resistant clone. In the course of ruxolitinib-dexamethasone treatment of patient 1145, hematologic improvement was observed. One CML patient (1408) in lymphatic blast crisis, which had failed both imatinib and dasatinib, achieved a 5-fold reduction in BCR-ABL1(T315I) transcript levels in bone marrow following 2 week axitinib treatment. Hence, even patients with transient clinical responses had a significant impact on the clonal evolution of the tumors and selection of mutations potentially contributing to drug resistance. Thus, comprehensive

genomic profiling of such patient cases is critical to evaluate the therapy effect and decipher mechanisms of drug sensitivity and resistance.

Table 9. Responses to DSRT-directed therapy based on ELN 2009 criteria

Patient	DSRT-guided treatment	Treatment duration (days)	Disease state at treatment start	Treatment response	Additional information	Time to progression (weeks)
252	Dasatinib	59	Relapsed, resistant disease	RD	Bone marrow blasts: 65-40-75%	8
560	Dasatinib-temsirolimus	34	Relapsed, remission	RD	Induction w. plerixafor-MAC	4
560	Dasatinib-sunitinib	5	Relapsed, resistant disease	RD	Blood blasts 34-0%	N/A
600	Dasatinib-sunitinib-temsirolimus	44	Relapsed, resistant disease	CRi		6
718	Sorafenib-clofarabine	63	Relapsed, resistant disease	Morphologic leukemia-free state	Hypoplasia	Not further follow-up
784	Dasatinib-sunitinib-temsirolimus	13	Resistant disease	RD	Bone marrow blasts: 70-35-85%	Not evaluable
800	Dasatinib-clofarabine-vinblastine	6	Resistant disease	Morphologic leukemia-free state	Hypoplasia	Hypoplasia, no disease progression
1145	Ruxolitinib-dexamethasone	48	Relapsed, resistant disease	RD	Hematologic improvement	6
1408	Axitinib	14	Resistant disease	N/E	5-fold reduction of BCR-ABL1(T315I) transcript levels	Allogeneic HSCT currently in CR

CRi-complete remission with incomplete platelet recovery; HSCT-hematopoietic stem cell transplant; RD-refractory disease; MAC-mitoxantrone, cytarabine, etoposide; morphologic leukemia-free state-bone marrow blasts < 5%, absence of blasts with Auer rods, absence of extramedullary disease, no hematologic recovery required; N/A-not available; N/E-not evaluable.

14.1 DSRT accurately recapitulates drug resistance *in vivo*

Patient 600 was a 54-year old male diagnosed with AML FAB M5 with normal karyotype and presence of an *FLT3*-ITD mutation. The patient failed 3 consecutive induction therapies and had a relapsed and refractory disease. Mononuclear cells isolated from the bone marrow of this patient were subjected to DSRT and in-depth molecular profiling to possibly identify molecular drivers and individualized therapy. The DSRT data showed strong selective responses for a number of tyrosine kinase inhibitors, including dasatinib and sunitinib, HSP90 inhibitors and rapalogs (Figure 23). Based on these results as well as availability of drugs the patient was treated with a combination of dasatinib, sunitinib and temsirolimus in an off-label compassionate use setting. This treatment strategy resulted in very quick reduction of bone marrow blasts and striking improvement of the clinical condition of the patient. Concurrently, a normalization of the bone marrow hematopoiesis ensued and the patient achieved complete remission with incomplete platelet recovery (Figure 24). The patient was able to leave the hospital for the first time in 6 months. However, the duration of therapy

response was approximately 30 days after which the patient relapsed. A new DSRT analysis of the relapsed cells indicated that there was a significant loss of sensitivity to not only the drugs the patient was treated with, but also to majority of the compounds in the DSRT panel. Hence, there was a perfect correlation between *in vivo* and *ex vivo* responses.

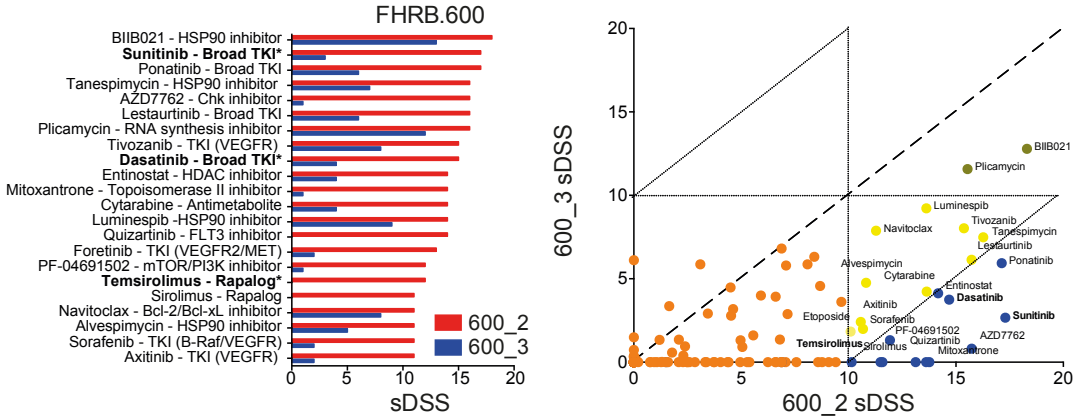


Figure 23. DSRT data and clinical implementation of DSRT results in a relapsed and refractory AML patient. The bar graph depicts the top leukemia selective compounds in patient sample 600_2 and the corresponding sensitivities to those agents in the relapsed sample 600_3. The correlation of the overall drug sensitivity profiles before and after relapse from dasatinib, sunitinib and temsirolimus treatment shows an omni-resistant phenotype. Drugs that had the strongest loss of sensitivity are marked in blue, intermediate loss in yellow and comparable sensitivity in green.

RNA sequencing data of RNA isolated from the 600_2 sample identified the presence of the fusion gene NUP98-NSD1 (Figure. 24) from a cryptic chromosomal rearrangement t(5;11)(q35;p15.5). Retrospective and prospective analysis showed the fusion transcript was present already at diagnosis and persisted following relapse from dasatinib, sunitinib and temsirolimus therapy, indicating that this fusion was involved in leukemia initiation. This genomic alteration[264] is relatively prominent in CN pediatric AML[265,266], but infrequent in adult AML[267]. Exome sequencing revealed a distinct clonal and subclonal evolution characterized by two different *FLT3*-ITD and four different *WT1* mutations (Figure. 24). To elucidate a model of disease progression, exome sequencing results were integrated with qPCR-measured alterations of the relative *FLT3*-ITD levels. The founding clone was defined by the fusion NUP98-NSD1 and mutations with stable tumor variant frequencies throughout the disease course. In addition, five other clones were detected. Clone 1, which was derived from the founding clone, was the dominant clone at diagnosis and was composed of mutations specific to the diagnostic sample (*FLG2* and *WDR5*) and *FLT3*-ITD #1. Clone 2 was also derived from the founding clone and was defined by *H2AFZ*, *SDR42E1*, *WT1* #1 and *FLT3*-ITD #2 mutations. In contrast, clone 3, 4, and 5 evolved from clone 2 by acquiring additional *WT1* mutations (#2, #3, and #4 respectively).

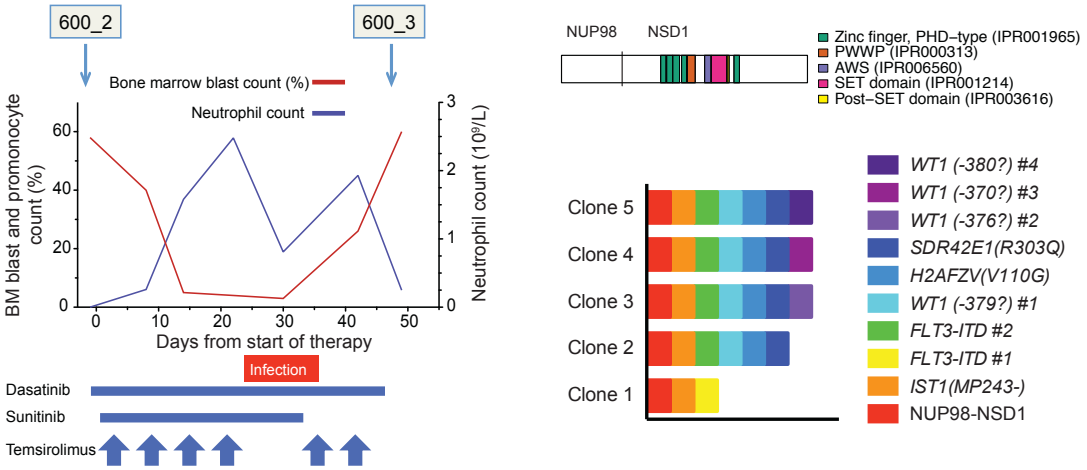


Figure 24. Clinical follow-up and clonal evolution analysis of the patient 600. The clinical graph shows alterations in blast and neutrophil counts in response to DSRT-guided treatment. The predicted protein structure of the NUP98-NSD1 fusion is also depicted and a summary plot of the composition of the distinct clones identified from the diagnosis, before and after DSRT-guided treatment samples with exome sequencing. Clones 1, 2 and 3 were present at diagnosis, clone 2, 3, 4 and 5 before relapse (600_2) and clone 2,3 and expanded clone 4 after relapse (600_3).

Since DSRT data of patient sample 600_2 demonstrated strong sensitivity to a number of FLT3 inhibitors (sunitinib, quizartinib, ponatinib, lestaurtinib, sorafenib) it is likely that FLT3 was one of the key disease drivers. However, dasatinib, sunitinib and temsirolimus treatment most probably led to the selection of a resistant clone, which albeit still containing the second *FLT3*-ITD conferred resistance to therapy. The drug resistance was accompanied with reduced phosphorylation of AKT, CHK2, CREB, ERK1/2, FAK, p38α, and STAT1. Genomic information did not fully elucidate the mechanism of drug resistance and disease progression as at relapse no novel mutations were detected, but rather a redistribution of the frequency of the already existing clones. Hence, other factors such as epigenetic modifications might have played a role in the development of the resistance phenotype in this patient.

14.2 DSRT and molecular profiling suggests mechanism of drug sensitivity and resistance

A 37-year old previously healthy woman presented with dysplastic changes in myelo- and thrombopoiesis. The patient was diagnosed with AML that was defined by a recurrent translocation t(11;19)(q23;p13.1) resulting in the MLL-ELL fusion gene. The patient relapsed following 3 rounds of conventional therapy. At that time point in the disease course (784_1), a bone marrow sample was taken for DSRT and molecular profiling. DSRT data revealed marked selective responses to MEK inhibitors, rapalogs, and a number of TKIs (e.g. dasatinib; Figure 25). Based on this data the patient was also treated with the dasatinib, sunitinib, and temsirolimus combination. This treatment regimen caused a rapid reduction of peripheral leukocytosis and bone marrow blasts, but the duration of the response was relatively short. The blast counts started to rise approximately 7 days following therapy commencement. The resistant cells (784_2) were also profiled with DSRT,

exome and RNA sequencing. The drug sensitivity profile of the 784_2 sample showed that the patient cells were not anymore sensitive to dasatinib and rapalogs, but exhibited comparable sensitivity to MEK inhibitors and ATP-competitive mTOR inhibitors (e.g. dactolisib and AZD8055) as the 784_1 sample (Figure 25). Strikingly, the resistant cells displayed sensitivity to several drugs and drug classes to which they were previously either not sensitive or had low level of sensitivity: BMS-754807 (IGF1R/Trk inhibitor), crizotinib (ALK inhibitor), tipifarnib (farnesyltransferase inhibitor), several topoisomerase II inhibitors and immunomodulators.

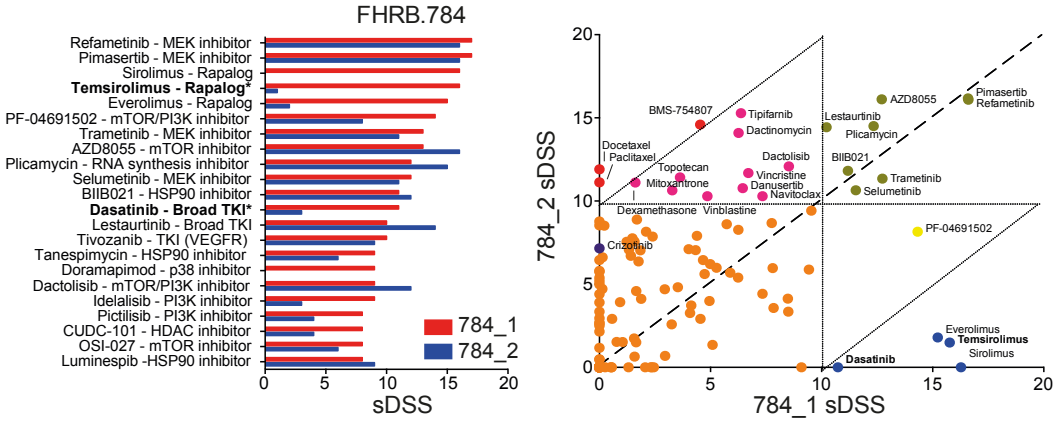


Figure 25. DSRT data and its clinical implementation in a relapsed and refractory AML patient. The bar graph depicts the top leukemia selective compounds in patient sample 784_1 and the corresponding sensitivities to those agents in the relapsed sample 784_2. The correlation of the overall drug sensitivity profiles before and after relapse from dasatinib, sunitinib and temsirolimus treatment is depicted on the right. Drugs that had the strongest loss of sensitivity are marked in blue, intermediate loss in yellow, retained sensitivity in green, and gain of sensitivity in pink and red.

The DSRT data before and after relapse was integrated with publicly available kinase inhibitor target specificity profiles[246] to perform a kinaddictome analysis, which revealed a significant shift in kinase addiction. In the pre-resistance sample the cells were deemed dependent on SRC family, PI3K and p38 mitogen-activated protein kinases, whereas that dependency was lost in the relapsed sample that gained addiction to ALK and Trk family RTKs. The loss of response to dasatinib, sunitinib and temsirolimus was accompanied with over 1,000-fold enrichment of two fusion transcripts ETV6-NTRK3 and STRN-ALK (Figure 26). This suggests that preexisting small subclones were selected with therapy and led to drug resistance. The fusion gene ETV6-NTRK3 encodes for the TEL-TrkC fusion protein with oncogenic properties, while the STRN-ALK was out of frame and could not code for a functional protein. In addition, the resistance phenotype was characterized with increase in the phosphorylation status of p70S6 kinase and CREB (Figure 26), implying a hyperactivation of mTORC1 that is a reported mechanism of resistance to rapamycin analogs[268].

The fusion gene MLL-ELL was detected in all samples from patient 784, suggesting it was the leukemia-initiating event. At diagnosis the patient harbored two distinct *FLT3*-ITD mutations, which were lost following induction chemotherapy. In addition a *WT1* mutation was also detected at diagnosis,

which was amplified by loss of heterozygosity (LOH) at the WT1 locus in following samples (784_1 and 784_2). In contrast, the frequencies of most other mutations observed in this patient remained stable throughout the disease progression, suggesting that they were derived from the founding clone. The altered sensitivity pattern to the dual IGF1R/TrkC inhibitor BMS-754807 in samples 784_1 and 784_2, suggests that the TEL-TrkC fusion protein became the disease driver in the resistant sample. Moreover, TEL-TrkC oncogenicity relies on IGF1R activation[269,270] and can cause hyperactivation of mTORC1[271,272]. Therefore, the mechanism of resistance to dasatinib, sunitinib and temsirolimus likely entailed TEL-TrkC dependent activation of the IGF1R signaling pathway, which stimulated mTORC1 hyperactivation. This postulate was reinforced by the detection of drug synergistic effects of BMS-754807 and the ATP-competitive mTOR inhibitor dactolisib in 784_2 cells (Figure 27). On the other hand, no such effects were observed between BMS-754807 and trametinib, a MEK inhibitor, indicating that an additional signal leading to MEK addiction was involved in the disease pathogenesis of this patient.

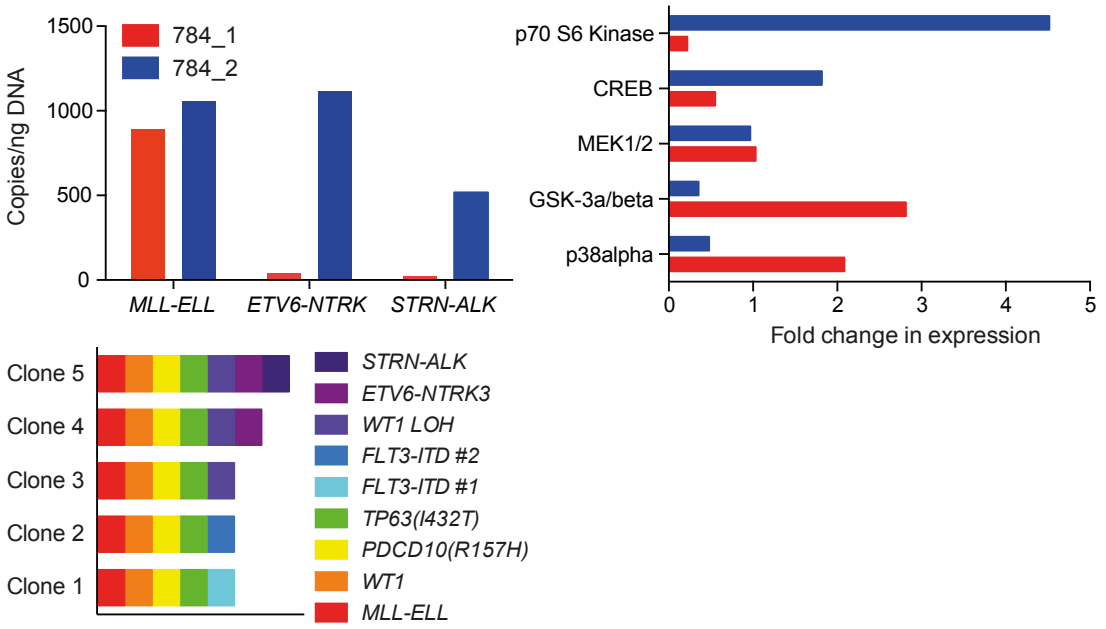


Figure 26. The drug resistance observed in patient 784 is defined by changes in clonal architecture and phosphoproteomics. Clones 1, 2 and 3 were present at diagnosis, clone 3 and low frequency of clones 4 and 5 before relapse (784_1) and clone 3 and expanded clones 4 and 5 after relapse (784_2).

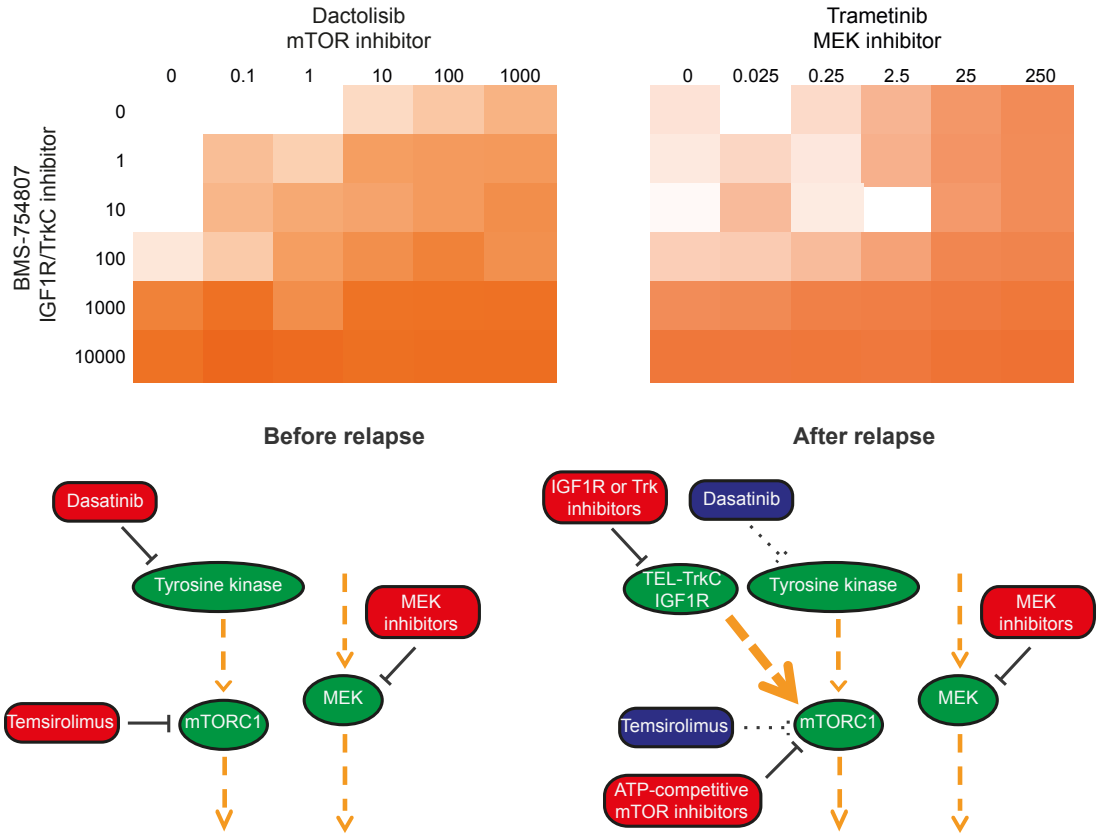


Figure 27. Oncogenic driver signals in the AML evolution of patient 784. Combinatorial treatment of BMS-754807 and either dactolisib or trametinib in drug resistant patient cells identified synergy between mTOR and IGF1R/TrkC inhibition. Integration of molecular and functional data enabled generation of a model of drug sensitivity and resistance. Red means sensitivity and blue means resistance.

14.3 Integration of functional and molecular data identifies mechanism of drug action

The direct targeting of BCR-ABL1(T315I) by axitinib was further validated biochemically. In line with the engineered Ba/F3 cell assays, axitinib selectively inhibited the ABL1(T315I) kinase activity with similar potency as VEGFR2[262,273], whereas its effects towards ABL1 were approximately 30-fold lower (Table 10).

Table 10. Kinase activity inhibition by axitinib measured with microfluidic mobility shift assay

Kinase	Axitinib K_i (nM)
VEGFR2	0.02 ± 0.004
ABL1(T315I)	0.1 ± 0.03
ABL1	3.8 ± 0.03

To determine the biding interactions and axitinib's preferential activity towards the mutated form of ABL1, X-ray crystallography was performed. The results showed that axitinib binds to ABL1 by forming 4 hydrogen bonds, 2 between the ABL1 kinase hinge segment and the indazole ring of axitinib and 2

between residues K271 and Y253 and axitinib's amide (Figure 28). In addition, the co-crystal structures of axitinib:ABL1 and axitinib:ABL1(T315I) surprisingly demonstrated a contrasting difference in the conformations of the activation loop (A-loop) of the kinases. Axitinib bound ABL1 when the kinase was in an inactive or DGF-out conformation, while ABL1(T315I) in an active or DFG-in conformation. Interestingly, axitinib binds its primary target VEGFR2 when the kinase adopts a DFG-out conformation (Figure 28). Furthermore, there was a difference in the P-loop conformation as well as large rotation of the axitinib:sulfur-indazole bond, which positioned the phenyl amide group in diverse binding pockets. Hence, in drug target interactions both the lock and the key can change.

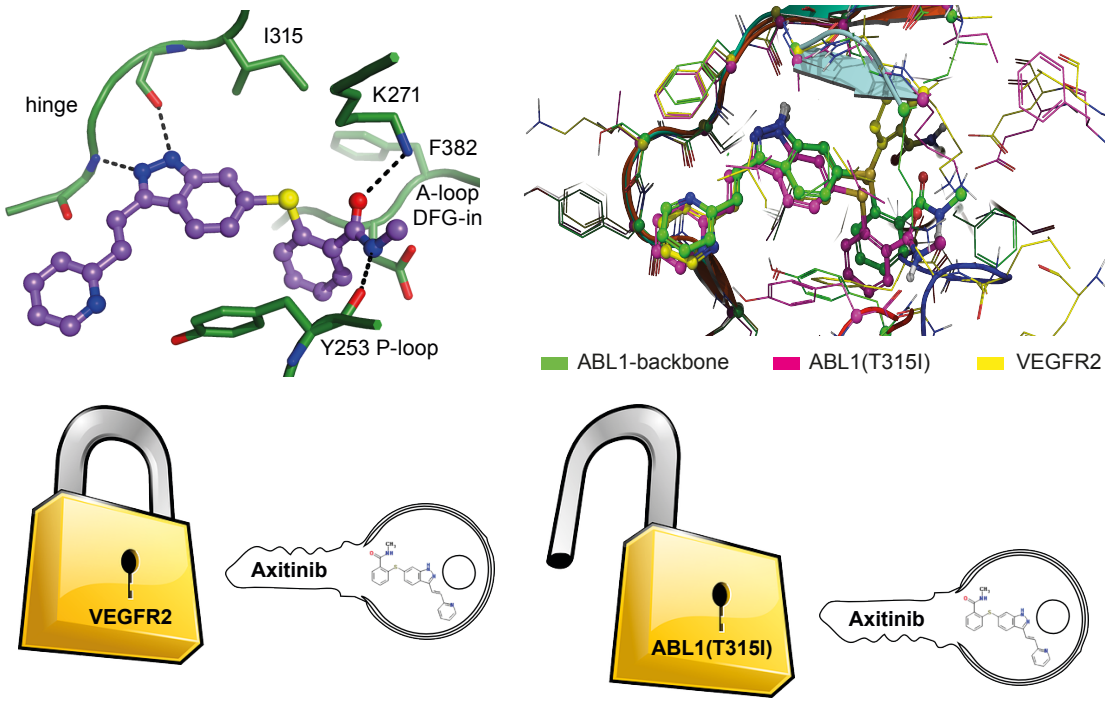


Figure 28. Co-crystal structures of axitinib bound to ABL1, ABL1(T315I) and VEGFR2 demonstrate different conformations of the inhibitor as well as the kinase.

Previous studies have shown that the T315I mutation stabilizes the DFG-in kinase conformation[274,275] and therefore the detected discrepancy in A-loop conformations of ABL1 and ABL1(T315I) most likely suggests an altered protein dynamics. Moreover, the increased potency towards the ABL1(T315I) kinase indicates that axitinib more optimally binds the DFG-in conformation of ABL1. This observation is supported with biochemical data that showed that axitinib more effectively inhibited autophosphorylated than non-phosphorylated ABL1(T315I) with K_i = 149 pM and 421 pM, respectively. Another surprising finding from the structural studies was that axitinib occupied a distinctive binding space in comparison to all other clinically available ABL1 inhibitors. Axitinib was not in as close proximity to the T315I residue and alpha-C helix in comparison to dasatinib and ponatinib. The reason that axitinib remains active towards the gatekeeper residue is that it does not form a hydrogen bond with the T315 in the first place and hence does not clash unfavorably with the I315 substitution unlike other ABL1

inhibitors[177]. These findings of gatekeeper-selective inhibition could be utilized for the development of a novel class of ABL1 targeted drugs.

A 35 year-old male was diagnosed with CML in lymphatic blast crisis and started on 600 mg imatinib monotherapy that led to a hematologic response (Figure 29). Two months later, the patient (FHRB.1408) had a hematologic relapse warranting a therapy switch to dasatinib (140 mg once-daily) to which no response was detected. At that point the kinase domain of BCR-ABL1 was sequenced, which revealed the presence of a T315I mutation in virtually all blast cells. The patient was subsequently treated with two cycles of CVAD (cyclophosphamide, vincristine, doxorubicin (also known as adriamycin), and dexamethasone) chemotherapy that reduced the relative T315I transcript levels to 20 percent. Mononuclear cells from this patient were then subjected to DSRT that detected leukemia selective sensitivity to immunomodulators, ATP-competitive mTOR inhibitors, ponatinib, axitinib, and MEK inhibitors (Figure 29). No response was seen to other ABL1 specific inhibitors.

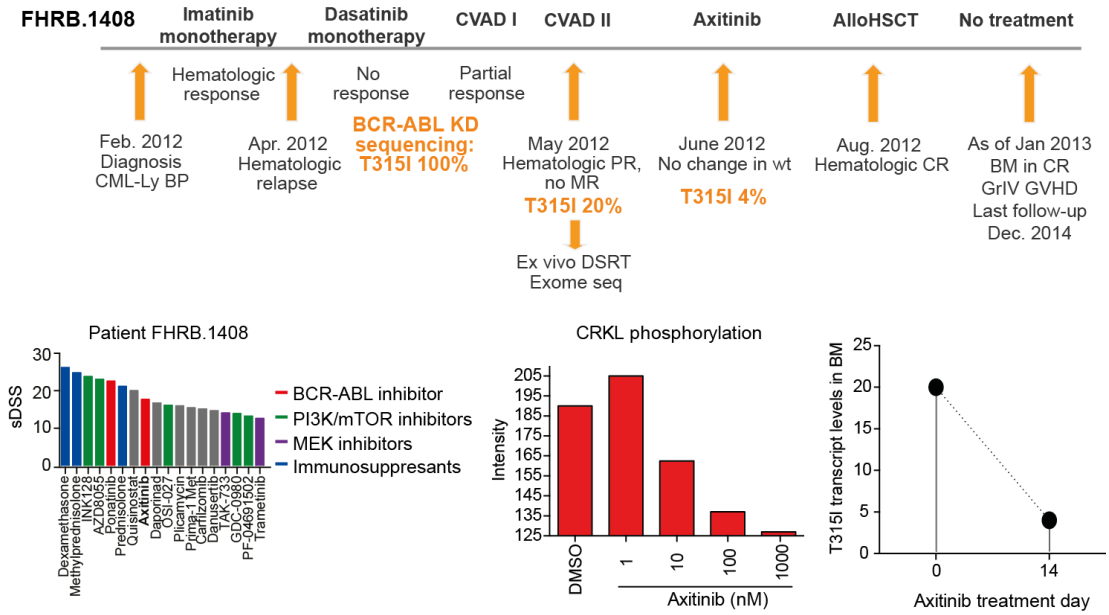


Figure 29. Clinical follow-up of patient 1408 and ex vivo and in vivo response to axitinib measured with DSRT, immunoblotting, and real-time quantitative PCR. BM-bone marrow; BP-blast phase; CR-complete response; GrIV GVHD-grade IV graft versus host disease; KD-kinase domain; Ly-lymphatic; MR-molecular response (PCR test for quantification of BCR-ABL1 in bone marrow cells); PR-partial response; seq-sequencing; wt-wild-type.

Since majority of the candidate drugs were not clinically available when the screen was performed, including ponatinib, the patient was treated in an off-label setting with the standard approved dose (5 mg twice daily) of axitinib for two weeks. Simultaneously, an immunoblotting analysis of CRKL (a surrogate marker of BCR-ABL1 activity) phosphorylation status in response to increasing concentrations of axitinib treatment of mononuclear cells from the same patient, prior to axitinib treatment, was performed. The results showed a dose-dependent reduction of CRKL phosphorylation in response to axitinib exposure, demonstrating that axitinib could inhibit BCR-ABL1 mediated signaling. The *in vivo* treatment of patient 1408 with axitinib led to the rapid

elimination of BCR-ABL1(T315I)-positive cells from the bone marrow as well as a 5-fold reduction of T315I transcript levels (Figure 29), implying that axitinib is capable of achieving on target efficacious responses in patients with BCR-ABL1(T315I) leukemias.

DISCUSSION and CONCLUSION

With the emerging challenges of translating cancer genome information into clinically actionable strategies and predicting and circumventing resistance to targeted drugs, it is clear that more effective ways are needed of testing cancers functionally. Comprehensive molecular and functional profiling efforts on large numbers of cancer cell lines were recently published[238,239,276-278] and the data from these studies showed to be valuable in linking molecular features of cancers to drug and compound sensitivities. However, the most powerful way of making these links would be to functionally and molecularly profile primary cancer samples and to follow one patient through different stages of disease to capture the plasticity of a cancer and emergence of resistance.

This thesis presented an 'Individualized Systems Medicine' strategy involving comprehensive *ex vivo* drug sensitivity testing of patient cancer cells combined with molecular and genomic profiling. This approach allowed us to functionally identify selective sensitivities of primary leukemia cells to a broad array of targeted anti-cancer agents, which could facilitate the identification of subgroups of patients that are likely to respond to a specific therapy and possibly even serve as a direct diagnostic tool. In addition, study I and II showed that the *ex vivo* drug sensitivities can be translated into efficacious therapeutic strategies and importantly, that one can follow emergence of resistance *in vivo* in the *ex vivo* assay and rapidly identify remaining or novel emerging drug sensitivities in the resistant patient cases. Associations between molecular and functional patterns further enable disease follow-up, determining clonal architecture and optimization of individualized therapy.

The overall goal of the thesis was to develop and implement a platform for functional and molecular characterization of cancer patient cells in order to gain a deeper understanding of disease pathogenetics as well as identify novel treatment strategies for individual patients. As disease models this thesis utilized primarily patient material from AML and Ph+ patients for both therapeutic and practical reasons. AML is intriguing to study, as even though it is a well-characterized disease[9,76,80,279,280], the current genomic and molecular knowledgebase has not facilitated identification of efficacious therapies and improvement of patient outcome. In contrast, since the early 2000's CML has become the poster child for the targeted drug revolution with the discovery and development of ABL1-specific inhibitors that significantly improved the outcome of patients from 5-year survival below 50% to 90%[281]. However, development of resistance to therapy, frequently due to the occurrence of point mutations in the kinase domain of ABL1, such as the gatekeeper T315I mutation, poses a significant clinical challenge. Practical benefits to investigating hematological malignancies include easy sampling and acquisition of large number of cells, possibility to generate sequential samples from the same patient and lower genetic complexity than most solid tumors[282].

The aim of the first study was to establish a drug testing approach to determine the sensitivity of hundreds of anti-cancer agents in primary AML cells with the aim of identifying novel treatment strategies and advancing the biological understanding of the disease. In the study, DSRT was applied to 28 AML patient samples and 7 healthy bone marrow control samples to identify selective subgroups to a range of molecularly targeted compounds. Drug sensitivity was quantified with a newly established drug sensitivity score that integrates the entire dose response data, as individual activity metrics do not sufficiently capture the complete dose response information[245]. SA I described the method in detail and showed additional applications of the drug sensitivity scoring approach, thus illustrating that DSS could provide complementary cognizance to genetic and epigenetic data on cancer phenotypes and addictions on a patient-by-patient basis.

Overall, molecularly targeted signal transduction-type inhibitors exhibited a less toxic response in control samples and a greater signal window towards sensitive samples than conventional chemotherapeutics. Compounds from the latter group seldom exhibited selective responses in sample subgroups, for example cytarabine and topoisomerase II inhibitors, but those are often more challenging to interpret and translate from *ex vivo* to *in vivo* setting. The response to mitotic inhibitors, for example, was strongly correlated with the mitotic activity of a sample in the assay plate, which likely has little predictive power of the response in an *in vivo* situation. With that notion, mitotic inhibitors were excluded from clustering data sets to avoid that the *ex vivo* mitotic activity would drive the grouping of the patient samples.

Among the signal transduction inhibitors that exhibited selective responses in subgroups of patient samples over control bone marrow samples were both major investigational oncology drug classes such as MEK inhibitors (although trametinib has since been approved for *BRAF* mutated melanoma), several tyrosine kinase inhibitors and ATP-competitive PI3K/AKT/mTOR pathway inhibitors, as well as approved rapamycin analog TORC1 inhibitors, JAK inhibitors and broad spectrum tyrosine kinase inhibitors such as dasatinib, sunitinib and sorafenib. Hence, the results point to the probability that already approved anti-cancer drugs, such as dasatinib (CML and Ph+ ALL), sunitinib (renal cell carcinoma and gastrointestinal tumors), and temsirolimus (renal cell cancer), could be explored for subsets of AML patients. Even though patients treated with DSRT-guided therapy did not achieve sustained cures, the clinical responses detected are promising in light of the fact that the patient cohort investigated was largely composed of relapsed and refractory patients with end stage disease. However, a fraction of patients that received DSRT-tailored treatment achieved remission levels that allowed the patients to undergo HSCT, which still remains as the only curative treatment for AML patients. The translation of ISM results to the clinic is an effective way to generate hypothesis to be evaluated in formal clinical trials for investigational as well as already approved drugs. Moreover, the ISM strategy could be utilized for identification of efficacious drug combinations according to correlating drug sensitivities.

Five functional taxonomic subgroups were defined based on unsupervised clustering of the *ex vivo* drug sensitivity profiles of the AML patient samples. Each subgroup was characterized with either sensitivity or non-sensitivity to anti-apoptotic agents, immunosuppressants, MEK, JAK, PI3K/AKT/mTOR and broad TK inhibitors. This analysis illustrates how unbiased functional profiling of cancer cells can facilitate matching drugs to individual patients. Furthermore, the drug responses were compared to the most frequent somatic mutational events in AML (as identified in the TCGA AML study[76]). While the sample cohort in this study was significantly smaller than the one used in the TCGA study, still certain phenotype to genotype links could be identified. For instance, sensitivity to FLT3 inhibitors was a strong predictor of activating *FLT3* mutations in subgroup V. However, it appears that it is not that simple and that patient samples falling in this group were also dependent on other TK signals targeted by inhibitors not inhibiting FLT3 such as dasatinib. This finding implies that there are cooperative signaling networks between oncogenic FLT3 signaling and one of TK whose combined targeting may provide an additional benefit in this patient population than FLT3 inhibitors alone. A proof of principle of this approach was shown with the patient case 600, where combinatorial treatment of dasatinib and sunitinib (targeting FLT3) led to a profound clinical response and subsequent complete remission following 3 failed induction chemotherapies. This example further demonstrates the power of the ISM and DSRT approaches to identify treatment strategies even for heavily refractory patients with no alternative treatment possibilities in the clinic.

MEK signaling has previously been suggested to play a role in AML pathogenesis[283-288]. Several MEK inhibitors have been in clinical trials for an AML indication, but none have been successful to date, possibly because no relevant patient stratification strategy was applied. Numerous trials have attempted to use the obvious predictive biomarker, the presence of *NRAS* mutations, and failed to yield positive results. In study I, 10 patient samples showed a highly selective sensitivity, indicating that the DSRT platform could be a way to identify candidate patients for MEK inhibitor therapy. While MEK addiction did not strongly link to sensitivity to a RTK inhibitor, a significant association was detected between activating *RAS* mutations or MLL fusions and MEK inhibitor sensitivity. However, approximately half of the sensitive patient samples did not harbor activating *RAS* mutations, suggesting that alternative or multiple upstream events may be involved in MEK addiction in AML such as baseline levels of ERK phosphorylation, constitutive activation of pathway components upstream of ERK, and expression of phosphatases (DUSP-4 and DUSP-6) and/or adaptor proteins (SPRY-2, SPRY-4, KSR1) that positively or negatively influence MAPK signaling[260,286,289]. Interestingly, recently *AHR* (encoding for aryl hydrocarbon receptor) expression was linked to MEK inhibitor sensitivity in *NRAS* mutant cell lines[238] and a transcriptional pathway signature was identified for selumetinib sensitivity in melanoma, colon, breast and lung tumor cell lines[290]. Further studies are needed to elucidate the complete mechanism of MEK addiction in primary AML cells, as *RAS* mutations alone do not fully explain the sensitivity to MEK inhibitors, and improved understanding of how to utilize MEK inhibitors presumably in combinations with other drugs for effective AML therapy.

Several inhibitors in the PI3K/AKT/mTOR pathway were identified as patient sample-selective compounds. Upregulation of the PI3K/AKT/mTOR pathway has been reported in AML patient samples and it is associated with poor prognosis[291,292]. Surprisingly, a strong correlation between selective sensitivity to the approved rapalog TORC1 inhibitors and ATP-competitive inhibitors of either the upstream PI3K or AKT classes of enzymes or by ATP-competitive mTOR inhibitors was not observed. Along those lines, very low background toxicity was detected with rapalogs while the ATP-competitive PI3K/AKT/mTOR inhibitors generally had significant toxicities and narrower therapeutic windows. This finding further shows the predictive value of the DRST in that it matches the clinical situation with these classes of compounds. Rapalogs are generally exhibiting low levels of toxicity *in vivo* compared to the other PI3K/AKT/mTOR inhibitors, which historically have had issues with toxicity in the clinic. The effects of mTOR inhibitors have been studied in an AML context before with conflicting findings. One group reported rapalog sensitivity with a resulting inhibition of also TORC2 and AKT[293], while another has reported that rapalog-induced TORC1 inhibition leads to activation of PI3K/AKT via upregulation of IGF1R signaling[272]. In our setting, the investigational IGF1R inhibitor BMS−754807 interestingly exhibited a response pattern very similar to ATP-competitive mTOR and PI3K inhibitors. Activated AKT has been detected and associated with poor prognosis in over 50% of AML patient samples[294] and activation of the AKT pathway was suggested to link to drug resistance in AML in others[138,295]. However the response to the AKT inhibitor MK-2206 was quite divergent than that of either rapalogs or PI3K or mTOR inhibitors. A study by Recher and colleagues reported *in vitro* anti-cancer activity of sirolimus in 4 out of 9 samples from patients with either refractory/relapsed or *de novo* AML[296], but rapalogs as monotherapy or in combination with high dose chemotherapy in relapsed and refractory AML patients have not proven clinically successful to date[297,298]. Hence, ongoing efforts are made to identify synergistic partners of rapalogs for efficacious AML therapy, for example as reported here rapalogs in combination with dasatinib or other potent broad tyrosine kinase inhibitors such as tivozanib, axitinib (that both appear to have related targets as dasatinib in AML), sunitinib or sorafenib.

In addition, study I showed that dasatinib, a broad tyrosine kinase inhibitor approved for use in BCR-ABL1-driven leukemias, stood out as one of the most selective RTK inhibitors in a subset of AML patient samples. ABL1 is not yet recognized as a significant driver kinase in AML and subsequently, dasatinib has not been studied extensively in the context of this disease. However, reports reveal a role for dasatinib in promoting ATRA-induced differentiation of AML cells[299] as well as prolonged *in vivo* differentiation of AML blasts carrying the t(8;21) translocation to mature, moderately functional neutrophils[300]. In addition, others have shown that dasatinib hinders growth of molecularly heterogeneous AML cell lines and that it caused dephosphorylation of hyperactivated Lyn kinase in AML patient samples[301]. Drug sensitivity profiles showed that dasatinib was selective and effective primarily in AML M5 diagnosed patient samples *ex vivo* and in combination with temsirolimus and sunitinib *in vivo* in an AML M5 patient with a *FLT3*-ITD mutation. In accordance, in a separate study, dasatinib sensitivity was only

seen in a patient diagnosed as AML M5b among a diverse panel of 8 AML patient samples[302]. The overall DSRT data does not indicate that the effect of dasatinib is due to single inhibition of Lyn or any other SRC family kinase, since other tested inhibitors targeting the same family of kinases (e.g. saracatinib) had different sensitivity profiles at the same time as several inhibitors that do not target SRC family kinases (e.g. tivozanib and axitinib) exhibited response patterns across the patient samples that were correlative to, but not as potent as the one of dasatinib. No obvious kinase target linked dasatinib to these inhibitors, so it is likely that these AML M5-selective kinase inhibitor sensitivities either depend on the inhibition of a combination of kinases or on non-kinase targets of these inhibitors. Nonetheless, dasatinib either as a single agent or in combination with chemotherapy or other targeted agents (e.g. temsirolimus or quizartinib) is a promising novel therapeutic strategy for relapsed and refractory AML M5 patients.

Overall the DSRT approach not only systematically validated candidate drugs that would otherwise be merely hypothesized based on genomic information, but also uncovered cancer cell addictions and dependencies that could not directly be deduced from molecular profiling data. The quantification of selective drug responses in our DSRT approach allowed for identification of drugs that exhibit leukemia-selective effects and hence likely have less toxicity. Comprehensive integrated analysis of phenotypes and genotypes in serial samples from individual patients facilitated optimization of personalized therapy, disease monitoring and follow up of the clonal composition of the cancer. Application of this approach to two case studies highlighted the significance of continual functional profiling of cancer cells in order to identify variations in drug sensitivities. On the one hand in patient 600 following relapse to dasatinib, sunitinib and temsirolimus treatment an omni-resistance phenotype was observed *ex vivo* that could not be completely explained by alterations in molecular profiles. On the other in patient 784 the response to that combinatorial therapy was lost likely due to enrichment of a clone harboring an ETV6-NTRK3 fusion gene encoding for an oncogenic tyrosine kinase that is a known driver in AML and other cancer types[303-305] and possibly could have been targeted by combinatorial inhibition of IGF1R and mTOR.

Besides to AML, the ISM/DSRT strategy was applied to Ph+ leukemias in study II, which led to the unexpected discovery that the VEGFR2 inhibitor axitinib also exhibits potent and selective BCR-ABL1(T315I) inhibitory activity. Currently, the only clinically available ABL1-specific inhibitor that has efficacy in this patient population is ponatinib[198,200], but vascular adverse events[202,306] and selection of resistant compound mutations[199,211] limit its clinical utility. Since the T315I mutation occurs in 15-20% of refractory CML and Ph+ ALL patients, there is a clear need for novel therapies with favorable safety profile. Integration of the functional *ex vivo* DSRT data with biochemical and structural analysis led to further validation that axitinib specifically targets BCR-ABL1(T315I) through a mutation-induced kinase conformational change distinct from its ABL1 and VEGFR2 binding modes. Given that axitinib occupies a very different binding space than other ABL1 inhibitors and has a

narrow target specificity profile, suggests that it will have a unique mutation vulnerability and favorable safety profile.

There have been previous reports of compounds exhibiting efficacy against the gatekeeper mutation in addition to ponatinib[197,261,307], but this effect was likely due to promiscuous nature[246] of the agents as their clinical use has been hindered by toxicity. Study II clearly illustrates that axitinib could be a beneficial addition to the ABL1 targeting toolbox due to its largely unique selectivity and binding to the gatekeeper mutation and limited polypharmacology. The distinct drug-target interaction features revealed by the axitinib-ABL1(T315I) binding could serve as an exemplar for development of even more effective gatekeeper-mutant selective inhibitors targeting ABL1 as well as other clinically important kinases, such as EGFR and KIT. Hence the findings of the study reflect a significant new concept where a kinase inhibitor selectively targets a subset of resistance mutations, which might have important implications in precision cancer medicine in the future. It is notable that clinical resistance mutations are occurring with virtually all TKIs to date and utilizing narrow targeted mutation selective inhibitors might be a simpler and less toxic approach to targeting new resistance mutations than developing individual inhibitors with an even broader spectrum.

Axitinib received regulatory approval in the USA and Europe in 2012 for the treatment of advanced renal cell carcinoma following failure to prior therapy. Clinical experience with the drug points to favorable safety profile with manageable side effects[308-310]. Therefore, study II stipulates that axitinib should be formally explored in patients with BCR-ABL1(T315I)-driven leukemia in a fast-track mode. Given the ABL1(T315I)-selective activity of axitinib, it is conceivable that T315I-negative clones might take over in response to axitinib treatment *in vivo*, especially since in patients not all blast cells might be T315I positive. Hence, combinations of axitinib with either other ABL1 inhibitors or inhibitors of downstream BCR-ABL1 effector signals (e.g. MEK, PI3K/mTOR, SMO) should provide a more complete clonal coverage and consequently more effective leukemia treatment. Since axitinib does not exhibit analogous adverse effects or overlapping toxicities[311,312] with other approved ABL1 drugs, it might be an ideal candidate for combinatorial approaches. However, the majority of ABL1 drugs on the market inactivate their metabolizing enzyme CYP3A4, which also metabolizes axitinib. It could therefore be challenging to determine dosing that avoids severe adverse effects of axitinib. Bosutinib, on the other hand, does not inhibit CYP3A4 and may therefore be a good combination partner with axitinib and might facilitate broad mutation coverage treatment. Previous research has shown that intermittent exposure of ABL1 inhibitors is sufficient for committing BCR-ABL1 positive cells to apoptosis and reduction of adverse effects such as pleural effusions[313,314]. Thus it is possible that the adverse effects of axitinib (on-target VEGFR-driven) could be reduced by non-continuous inhibition since both on- and off-target effects of inhibiting VEGFRs typically come from persistent inhibition[315].

While the ISM strategy is a powerful method for customizing patient treatment and identification of biomarkers that explain drug responses, the number and

types of drugs currently available limit the approach. The oncology drug collection used in these studies is heavily biased towards kinase inhibitors, which have an inherent problem of short effective response duration and rapid onset of resistance that is often very challenging if not impossible to overcome. However, the DSRT platform is adaptable to different types of drugs and drug classes that can be fed in as they become available providing the opportunity to interrogate a vast array of targets. Despite the use of primary patient material in this thesis, it is likely that some of the tumor complexity in terms of cell differentiation and tumor microenvironment is lost on plastic. Hence, improved culture conditions that preserve the state of the cancer cells and take into account the tumor environment might enhance the predictive power of *ex vivo* drug sensitivity testing approaches as well as provide additional insights into disease biology. Furthermore, to validate that this approach accurately predicts drug responses and can be of significant benefit to patients it will need to be evaluated in a formal clinical trial. This would be contingent on several pharmaceutical companies providing their drugs for a bucket trial design, which is not an easy or simple feat. As most cancer patients are treated with drug combinations, the DSRT pipeline will also need to be adapted to systematic drug combination testing as well as means to assess or predict toxicity of individual as well as combined drugs. While single agents are easier to implement and verify, often no long-term responses are achieved. In contrast, combinatorial therapy might produce longer-lasting responses, but the challenge remains on how best to design it whether based on correlative drug responses from profiling data, addition of chemotherapy to an experimental drug or based on distinct mode of action.

In conclusion, pharmacopeia-wide drug sensitivity and resistance testing led to the identification of several classes of signal transduction inhibitors that exhibited selective activity in distinct and partly overlapping subsets of AML patient samples. Network analysis of associated inhibitors and their target selectivities allowed us to reveal clues to cancer molecular pathogenesis and driver addictions in the patient samples. It is likely that the DSRT data integrated with molecular profiling and clinical information will become a powerful tool to link selective drug responses to clinically actionable markers, patient stratification and novel therapeutic strategies for managing and curing aggressive cancers. This integrated systems biology approach is an effective hypothesis generator to be explored in future clinical trials. The data presented in this thesis further shows the power of unbiased functional profiling of patient derived cancer cells in identifying novel drug-target and drug-patient interactions with broad implications. Moreover, a novel functional classification of AML was introduced that can be further refined with a larger cohort of patients and be of benefit for improved patient stratification and drug-patient matching. The studies included in this thesis emphasize the benefit of drug repurposing and investigating new uses of already existing drugs as well as illustrate that DSRT can serve as a drug positioning and de-risking tool of investigational compounds. A future goal will be the systematic testing of the diagnostic value of the DSRT approach in identifying sensitive patient subgroups and possible adverse effects in a clinical setting as well to evaluate whether DSRT-based therapy as first line rather then chemotherapy might prevent relapse and progressive disease evolution. In addition, adapting

the platform to testing drug combinations *ex vivo,* developing more comprehensive readouts as well as detecting the impact of drug treatment on cell signaling and clonal composition might improve the predictive power of the DSRT.

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REFERENCES

1 Patel, M. N., Halling-Brown, M. D., Tym, J. E., Workman, P. & Al-Lazikani, B. Objective assessment of cancer genes for drug discovery. *Nat Rev Drug Discov* **12**, 35-50, (2013).

2 Chin, L., Hahn, W. C., Getz, G. & Meyerson, M. Making sense of cancer genomic data. *Genes Dev* **25**, 534-555, (2011).

3 Garraway, L. A. & Janne, P. A. Circumventing cancer drug resistance in the era of personalized medicine. *Cancer Discov* **2**, 214-226, (2012).

4 Greaves, M. & Maley, C. C. Clonal evolution in cancer. *Nature* **481**, 306-313, (2012).

5 Cox, A. D., Fesik, S. W., Kimmelman, A. C., Luo, J. & Der, C. J. Drugging the undruggable RAS: Mission possible? *Nat Rev Drug Discov* **13**, 828-851, (2014).

6 Garraway, L. A. & Lander, E. S. Lessons from the cancer genome. *Cell* **153**, 17-37, (2013).

7 Workman, P., Al-Lazikani, B. & Clarke, P. A. Genome-based cancer therapeutics: targets, kinase drug resistance and future strategies for precision oncology. *Curr Opin Pharmacol* **13**, 486-496, (2013).

8 Burnett, K. A., Tallman, M.S. Advances in the Molecular Biology and Treatment of Adults with Acute Myeloid Leukemia. *Am Soc Clin Oncol* **1092-9118/ 10/ 1-10**, 225-230, (2011).

9 Dohner, H. *et al.* Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* **115**, 453-474, (2010).

10 Swerdlow, S. H. *et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Fourth Edition*. Vol. 2 (IARC Press, 2008).

11 Frohling, S., Scholl, C., Gilliland, D. G. & Levine, R. L. Genetics of myeloid malignancies: pathogenetic and clinical implications. *J Clin Oncol* **23**, 6285-6295, (2005).

12 Marcucci, G., Haferlach, T. & Dohner, H. Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. *J Clin Oncol* **29**, 475-486, (2011).

13 Kumar, C. C. Genetic abnormalities and challenges in the treatment of acute myeloid leukemia. *Genes Cancer* **2**, 95-107, (2011).

14 Estey, E. H. Acute myeloid leukemia: 2012 update on diagnosis, risk stratification, and management. *Am J Hematol* **87**, 89-99, (2012).

15 Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2015. *CA Cancer J Clin* **65**, 5-29, (2015).

16 Hasserjian, R. P. Acute myeloid leukemia: advances in diagnosis and classification. *Int J Lab Hematol* **35**, 358-366, (2013).

17 Tallman, M. S., Gilliland, D. G. & Rowe, J. M. Drug therapy for acute myeloid leukemia. *Blood* **106**, 1154-1163, (2005).

18 Haferlach, T. Molecular genetic pathways as therapeutic targets in acute myeloid leukemia. *Hematology Am Soc Hematol Educ Program*, 400-411, (2008).

19 Estey, E. & Dohner, H. Acute myeloid leukaemia. *Lancet* **368**, 1894-1907, (2006).

20 Erba, H. P. Has there been progress in the treatment of older patients with acute myeloid leukemia? *Best Pract Res Clin Haematol* **23**, 495-501, (2010).

21 Schlenk, R. F. *et al.* Intensive consolidation versus oral maintenance therapy in patients 61 years or older with acute myeloid leukemia in first remission: results of second randomization of the AML HD98-B treatment Trial. *Leukemia* **20**, 748-750, (2006).

22 Kohrt, H. E. & Coutre, S. E. Optimizing therapy for acute myeloid leukemia. *J Natl Compr Canc Netw* **6**, 1003-1016, (2008).

23 Deschler, B., de Witte, T., Mertelsmann, R. & Lubbert, M. Treatment decision-making for older patients with high-risk myelodysplastic syndrome or acute myeloid leukemia: problems and approaches. *Haematologica* **91**, 1513-1522, (2006).

24 Kell, J. Treatment of relapsed acute myeloid leukaemia. *Rev Recent Clin Trials* **1**, 103-111, (2006).

25 Shipley, J. L. & Butera, J. N. Acute myelogenous leukemia. *Exp Hematol* **37**, 649-658, (2009).

26 Pollyea, D. A., Kohrt, H. E. & Medeiros, B. C. Acute myeloid leukaemia in the elderly: a review. *Br J Haematol* **152**, 524-542, (2011).
27 Grimwade, D. *et al.* Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. *Blood* **116**, 354-365, (2010).
28 Burnett, A., Wetzler, M. & Lowenberg, B. Therapeutic advances in acute myeloid leukemia. *J Clin Oncol* **29**, 487-494, (2011).
29 Thomas, X. Chemotherapy of acute leukemia in adults. *Expert Opin Pharmacother* **10**, 221-237, (2009).
30 Lowenberg, B. *et al.* Gemtuzumab ozogamicin as postremission treatment in AML at 60 years of age or more: results of a multicenter phase 3 study. *Blood* **115**, 2586-2591, (2010).
31 A systematic collaborative overview of randomized trials comparing idarubicin with daunorubicin (or other anthracyclines) as induction therapy for acute myeloid leukaemia. AML Collaborative Group. *Br J Haematol* **103**, 100-109, (1998).
32 Malek, S. N. Update on the Molecular Biology of Acute Myelogenous Leukemia: Clinacal Implications. *Am Soc Clin Oncol* **1092-9118/10/1-10**, 231-236, (2011).
33 Bennett, J. M. *et al.* Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol* **33**, 451-458, (1976).
34 Bennett, J. M. *et al.* Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med* **103**, 620-625, (1985).
35 Harris, N. L. *et al.* The World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues. Report of the Clinical Advisory Committee meeting, Airlie House, Virginia, November, 1997. *Ann Oncol* **10**, 1419-1432, (1999).
36 Martens, J. H. & Stunnenberg, H. G. The molecular signature of oncofusion proteins in acute myeloid leukemia. *FEBS Lett* **584**, 2662-2669, (2010).
37 Mrozek, K. *et al.* Prognostic significance of the European LeukemiaNet standardized system for reporting cytogenetic and molecular alterations in adults with acute myeloid leukemia. *J Clin Oncol* **30**, 4515-4523, (2012).
38 Speck, N. A. & Gilliland, D. G. Core-binding factors in haematopoiesis and leukaemia. *Nat Rev Cancer* **2**, 502-513, (2002).
39 Paschka, P. *et al.* Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. *J Clin Oncol* **24**, 3904-3911, (2006).
40 Schnittger, S. *et al.* KIT-D816 mutations in AML1-ETO-positive AML are associated with impaired event-free and overall survival. *Blood* **107**, 1791-1799, (2006).
41 Kuchenbauer, F. *et al.* Impact of FLT3 mutations and promyelocytic leukaemia-breakpoint on clinical characteristics and prognosis in acute promyelocytic leukaemia. *Br J Haematol* **130**, 196-202, (2005).
42 Lin, R. J. & Evans, R. M. Acquisition of oncogenic potential by RAR chimeras in acute promyelocytic leukemia through formation of homodimers. *Mol Cell* **5**, 821-830, (2000).
43 Douer, D. & Tallman, M. S. Arsenic trioxide: new clinical experience with an old medication in hematologic malignancies. *J Clin Oncol* **23**, 2396-2410, (2005).
44 Lallemand-Breitenbach, V. *et al.* Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. *Nat Cell Biol* **10**, 547-555, (2008).
45 Estey, E. H. Acute myeloid leukemia: 2014 update on risk-stratification and management. *Am J Hematol* **89**, 1063-1081, (2014).
46 Marcucci, G., Mrozek, K. & Bloomfield, C. D. Molecular heterogeneity and prognostic biomarkers in adults with acute myeloid leukemia and normal cytogenetics. *Curr Opin Hematol* **12**, 68-75, (2005).
47 Mrozek, K., Dohner, H. & Bloomfield, C. D. Influence of new molecular prognostic markers in patients with karyotypically normal acute myeloid leukemia: recent advances. *Curr Opin Hematol* **14**, 106-114, (2007).
48 Gilliland, D. G. & Griffin, J. D. The roles of FLT3 in hematopoiesis and leukemia. *Blood* **100**, 1532-1542, (2002).

49 Small, D. Targeting FLT3 for the treatment of leukemia. *Semin Hematol* **45**, S17-21, (2008).
50 Stirewalt, D. L. & Radich, J. P. The role of FLT3 in haematopoietic malignancies. *Nat Rev Cancer* **3**, 650-665, (2003).
51 Tyner, J. W. *et al.* High-throughput sequencing screen reveals novel, transforming RAS mutations in myeloid leukemia patients. *Blood* **113**, 1749-1755, (2009).
52 Ernst, P., Wang, J. & Korsmeyer, S. J. The role of MLL in hematopoiesis and leukemia. *Curr Opin Hematol* **9**, 282-287, (2002).
53 Nerlov, C. C/EBPalpha mutations in acute myeloid leukaemias. *Nat Rev Cancer* **4**, 394-400, (2004).
54 Grisendi, S., Mecucci, C., Falini, B. & Pandolfi, P. P. Nucleophosmin and cancer. *Nat Rev Cancer* **6**, 493-505, (2006).
55 Ley, T. J. *et al.* DNMT3A mutations in acute myeloid leukemia. *N Engl J Med* **363**, 2424-2433, (2010).
56 Gilliland, D. G., Jordan, C. T. & Felix, C. A. The molecular basis of leukemia. *Hematology Am Soc Hematol Educ Program*, 80-97, (2004).
57 Renneville, A. *et al.* Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia* **22**, 915-931, (2008).
58 Ishikawa, Y. *et al.* Comprehensive analysis of cooperative gene mutations between class I and class II in de novo acute myeloid leukemia. *Eur J Haematol* **83**, 90-98, (2009).
59 Falini, B. *et al.* Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med* **352**, 254-266, (2005).
60 Pasqualucci, L. *et al.* Mutated nucleophosmin detects clonal multilineage involvement in acute myeloid leukemia: Impact on WHO classification. *Blood* **108**, 4146-4155, (2006).
61 Thiede, C. *et al.* Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood* **107**, 4011-4020, (2006).
62 Schlenk, R. F. *et al.* Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med* **358**, 1909-1918, (2008).
63 Haferlach, C. *et al.* AML with mutated NPM1 carrying a normal or aberrant karyotype show overlapping biologic, pathologic, immunophenotypic, and prognostic features. *Blood* **114**, 3024-3032, (2009).
64 Dohner, H. & Gaidzik, V. I. Impact of genetic features on treatment decisions in AML. *Hematology Am Soc Hematol Educ Program* **2011**, 36-42, (2011).
65 Whitman, S. P. *et al.* FLT3 D835/I836 mutations are associated with poor disease-free survival and a distinct gene-expression signature among younger adults with de novo cytogenetically normal acute myeloid leukemia lacking FLT3 internal tandem duplications. *Blood* **111**, 1552-1559, (2008).
66 Bacher, U., Haferlach, C., Kern, W., Haferlach, T. & Schnittger, S. Prognostic relevance of FLT3-TKD mutations in AML: the combination matters--an analysis of 3082 patients. *Blood* **111**, 2527-2537, (2008).
67 Smith, C. C. *et al.* Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia. *Nature* **485**, 260-263, (2012).
68 Thiede, C. *et al.* Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood* **99**, 4326-4335, (2002).
69 Schnittger, S. *et al.* Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood* **100**, 59-66, (2002).
70 Port, M. *et al.* Prognostic significance of FLT3 internal tandem duplication, nucleophosmin 1, and CEBPA gene mutations for acute myeloid leukemia patients with normal karyotype and younger than 60 years: a systematic review and meta-analysis. *Ann Hematol* **93**, 1279-1286, (2014).
71 Radomska, H. S. *et al.* CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol Cell Biol* **18**, 4301-4314, (1998).

72 Koschmieder, S., Halmos, B., Levantini, E. & Tenen, D. G. Dysregulation of the C/EBPalpha differentiation pathway in human cancer. *J Clin Oncol* **27**, 619-628, (2009).
73 Wouters, B. J. *et al.* Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. *Blood* **113**, 3088-3091, (2009).
74 Bereshchenko, O. *et al.* Hematopoietic stem cell expansion precedes the generation of committed myeloid leukemia-initiating cells in C/EBPalpha mutant AML. *Cancer Cell* **16**, 390-400, (2009).
75 Chan, S. M. & Majeti, R. Role of DNMT3A, TET2, and IDH1/2 mutations in pre-leukemic stem cells in acute myeloid leukemia. *Int J Hematol* **98**, 648-657, (2013).
76 Cancer Genome Atlas Research, N. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med* **368**, 2059-2074, (2013).
77 Yan, X. J. *et al.* Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nat Genet* **43**, 309-315, (2011).
78 Im, A. P. *et al.* DNMT3A and IDH mutations in acute myeloid leukemia and other myeloid malignancies: associations with prognosis and potential treatment strategies. *Leukemia* **28**, 1774-1783, (2014).
79 Hajkova, H. *et al.* Decreased DNA methylation in acute myeloid leukemia patients with DNMT3A mutations and prognostic implications of DNA methylation. *Leuk Res* **36**, 1128-1133, (2012).
80 Figueroa, M. E. *et al.* DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. *Cancer Cell* **17**, 13-27, (2010).
81 Schoofs, T., Berdel, W. E. & Muller-Tidow, C. Origins of aberrant DNA methylation in acute myeloid leukemia. *Leukemia* **28**, 1-14, (2014).
82 Patel, J. P. *et al.* Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. *N Engl J Med* **366**, 1079-1089, (2012).
83 Flotho, C. *et al.* The DNA methyltransferase inhibitors azacitidine, decitabine and zebularine exert differential effects on cancer gene expression in acute myeloid leukemia cells. *Leukemia* **23**, 1019-1028, (2009).
84 Pinto, A. *et al.* 5-Aza-2'-deoxycytidine induces terminal differentiation of leukemic blasts from patients with acute myeloid leukemias. *Blood* **64**, 922-929, (1984).
85 Curik, N. *et al.* 5-azacitidine in aggressive myelodysplastic syndromes regulates chromatin structure at PU.1 gene and cell differentiation capacity. *Leukemia* **26**, 1804-1811, (2012).
86 Stresemann, C. & Lyko, F. Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *Int J Cancer* **123**, 8-13, (2008).
87 Foulks, J. M. *et al.* Epigenetic drug discovery: targeting DNA methyltransferases. *J Biomol Screen* **17**, 2-17, (2012).
88 Blum, W. *et al.* Clinical response and miR-29b predictive significance in older AML patients treated with a 10-day schedule of decitabine. *Proc Natl Acad Sci U S A* **107**, 7473-7478, (2010).
89 Kantarjian, H. M. *et al.* Multicenter, randomized, open-label, phase III trial of decitabine versus patient choice, with physician advice, of either supportive care or low-dose cytarabine for the treatment of older patients with newly diagnosed acute myeloid leukemia. *J Clin Oncol* **30**, 2670-2677, (2012).
90 Fenaux, P. *et al.* Azacitidine prolongs overall survival compared with conventional care regimens in elderly patients with low bone marrow blast count acute myeloid leukemia. *J Clin Oncol* **28**, 562-569, (2010).
91 DiNardo, C. D. *et al.* Lack of association of IDH1, IDH2 and DNMT3A mutations with outcome in older patients with acute myeloid leukemia treated with hypomethylating agents. *Leuk Lymphoma* **55**, 1925-1929, (2014).
92 Yan, H. *et al.* IDH1 and IDH2 mutations in gliomas. *N Engl J Med* **360**, 765-773, (2009).
93 Marcucci, G. *et al.* IDH1 and IDH2 gene mutations identify novel molecular subsets within de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *J Clin Oncol* **28**, 2348-2355, (2010).

94 Paschka, P. *et al.* IDH1 and IDH2 mutations are frequent genetic alterations in acute myeloid leukemia and confer adverse prognosis in cytogenetically normal acute myeloid leukemia with NPM1 mutation without FLT3 internal tandem duplication. *J Clin Oncol* **28**, 3636-3643, (2010).
95 Schnittger, S. *et al.* IDH1 mutations are detected in 6.6% of 1414 AML patients and are associated with intermediate risk karyotype and unfavorable prognosis in adults younger than 60 years and unmutated NPM1 status. *Blood* **116**, 5486-5496, (2010).
96 Dang, L. *et al.* Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* **462**, 739-744, (2009).
97 Ward, P. S. *et al.* The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* **17**, 225-234, (2010).
98 Figueroa, M. E. *et al.* Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* **18**, 553-567, (2010).
99 Popovici-Muller, J. *et al.* Pyrazolo[1,5-a]pyrimidine-based inhibitors of HCV polymerase. *Bioorganic & medicinal chemistry letters* **19**, 6331-6336, (2009).
100 Rohle, D. *et al.* An inhibitor of mutant IDH1 delays growth and promotes differentiation of glioma cells. *Science* **340**, 626-630, (2013).
101 Wang, F. *et al.* Targeted inhibition of mutant IDH2 in leukemia cells induces cellular differentiation. *Science* **340**, 622-626, (2013).
102 Delhommeau, F. *et al.* Mutation in TET2 in myeloid cancers. *N Engl J Med* **360**, 2289-2301, (2009).
103 Ito, S. *et al.* Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* **466**, 1129-1133, (2010).
104 Ko, M. *et al.* Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature* **468**, 839-843, (2010).
105 Damm, F. *et al.* TET2 mutations in cytogenetically normal acute myeloid leukemia: clinical implications and evolutionary patterns. *Genes Chromosomes Cancer* **53**, 824-832, (2014).
106 Krauth, M. T. *et al.* WT1 mutations are secondary events in AML, show varying frequencies and impact on prognosis between genetic subgroups. *Leukemia*, (2014).
107 Yang, L., Han, Y., Suarez Saiz, F. & Minden, M. D. A tumor suppressor and oncogene: the WT1 story. *Leukemia* **21**, 868-876, (2007).
108 Paschka, P. *et al.* Wilms' tumor 1 gene mutations independently predict poor outcome in adults with cytogenetically normal acute myeloid leukemia: a cancer and leukemia group B study. *J Clin Oncol* **26**, 4595-4602, (2008).
109 Virappane, P. *et al.* Mutation of the Wilms' tumor 1 gene is a poor prognostic factor associated with chemotherapy resistance in normal karyotype acute myeloid leukemia: the United Kingdom Medical Research Council Adult Leukaemia Working Party. *J Clin Oncol* **26**, 5429-5435, (2008).
110 Gaidzik, V. I. *et al.* Prognostic impact of WT1 mutations in cytogenetically normal acute myeloid leukemia: a study of the German-Austrian AML Study Group. *Blood* **113**, 4505-4511, (2009).
111 Rampal, R. *et al.* DNA hydroxymethylation profiling reveals that WT1 mutations result in loss of TET2 function in acute myeloid leukemia. *Cell Rep* **9**, 1841-1855, (2014).
112 Medeiros, B. C., Othus, M., Fang, M., Roulston, D. & Appelbaum, F. R. Prognostic impact of monosomal karyotype in young adult and elderly acute myeloid leukemia: the Southwest Oncology Group (SWOG) experience. *Blood* **116**, 2224-2228, (2010).
113 Breems, D. A. *et al.* Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. *J Clin Oncol* **26**, 4791-4797, (2008).
114 Christiansen, D. H., Andersen, M. K. & Pedersen-Bjergaard, J. Mutations with loss of heterozygosity of p53 are common in therapy-related myelodysplasia and acute myeloid leukemia after exposure to alkylating agents and significantly associated with deletion or loss of 5q, a complex karyotype, and a poor prognosis. *J Clin Oncol* **19**, 1405-1413, (2001).
115 Rucker, F. G. *et al.* TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome. *Blood* **119**, 2114-2121, (2012).

116 Welch, J. S. *et al.* The origin and evolution of mutations in acute myeloid leukemia. *Cell* **150**, 264-278, (2012).
117 Grove, C. S. & Vassiliou, G. S. Acute myeloid leukaemia: a paradigm for the clonal evolution of cancer? *Dis Model Mech* **7**, 941-951, (2014).
118 Wartman, L. D. *et al.* Sequencing a mouse acute promyelocytic leukemia genome reveals genetic events relevant for disease progression. *J Clin Invest* **121**, 1445-1455, (2011).
119 Mupo, A. *et al.* A powerful molecular synergy between mutant Nucleophosmin and Flt3-ITD drives acute myeloid leukemia in mice. *Leukemia* **27**, 1917-1920, (2013).
120 Anderson, K. *et al.* Genetic variegation of clonal architecture and propagating cells in leukaemia. *Nature* **469**, 356-361, (2011).
121 Ding, L. *et al.* Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature* **481**, 506-510, (2012).
122 Kronke, J. *et al.* Clonal evolution in relapsed NPM1-mutated acute myeloid leukemia. *Blood* **122**, 100-108, (2013).
123 Parkin, B. *et al.* Clonal evolution and devolution after chemotherapy in adult acute myelogenous leukemia. *Blood* **121**, 369-377, (2013).
124 Christiansen, D. H., Andersen, M. K., Desta, F. & Pedersen-Bjergaard, J. Mutations of genes in the receptor tyrosine kinase (RTK)/RAS-BRAF signal transduction pathway in therapy-related myelodysplasia and acute myeloid leukemia. *Leukemia* **19**, 2232-2240, (2005).
125 Cortes, J. E. *et al.* Phase 1 AML study of AC220, a potent and selective second generation FLT3 receptor tyrosine kinase inhibitor. *Blood (ASH Ann Meet Abstr)* **112:Absract 767**, (2008).
126 Cortes, J. E. *et al.* Final results of a phase 2 open-label, monotherapy efficacy and safety study of quizartinib (AC220) in patients ≥ 60 years of age with FLT ITD positive or negative relapsed/refractory acute myeloid leukemia. *Blood (ASH Ann Meet Abstr)* **120:Abstract 48**, (2012).
127 Sexauer, A. *et al.* Terminal myeloid differentiation in vivo is induced by FLT3 inhibition in FLT3/ITD AML. *Blood* **120**, 4205-4214, (2012).
128 Zhang, W. *et al.* Mutant FLT3: a direct target of sorafenib in acute myelogenous leukemia. *J Natl Cancer Inst* **100**, 184-198, (2008).
129 Borthakur, G. *et al.* Phase I study of sorafenib in patients with refractory or relapsed acute leukemias. *Haematologica* **96**, 62-68, (2011).
130 Fischer, T. *et al.* Phase IIB trial of oral Midostaurin (PKC412), the FMS-like tyrosine kinase 3 receptor (FLT3) and multi-targeted kinase inhibitor, in patients with acute myeloid leukemia and high-risk myelodysplastic syndrome with either wild-type or mutated FLT3. *J Clin Oncol* **28**, 4339-4345, (2010).
131 Galanis, A. *et al.* Crenolanib is a potent inhibitor of FLT3 with activity against resistance-conferring point mutants. *Blood* **123**, 94-100, (2014).
132 Smith, C. C. *et al.* Crenolanib is a selective type I pan-FLT3 inhibitor. *Proc Natl Acad Sci U S A* **111**, 5319-5324, (2014).
133 Kiyoi, H. Flt3 Inhibitors: Recent Advances and Problems for Clinical Application. *Nagoya J Med Sci* **77**, 7-17, (2015).
134 Dos Santos, C. *et al.* The Src and c-Kit kinase inhibitor dasatinib enhances p53-mediated targeting of human acute myeloid leukemia stem cells by chemotherapeutic agents. *Blood* **122**, 1900-1913, (2013).
135 McCubrey, J. A. *et al.* Targeting survival cascades induced by activation of Ras/Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways for effective leukemia therapy. *Leukemia* **22**, 708-722, (2008).
136 Adjei, A. A. *et al.* Phase I pharmacokinetic and pharmacodynamic study of the oral, small-molecule mitogen-activated protein kinase kinase 1/2 inhibitor AZD6244 (ARRY-142886) in patients with advanced cancers. *J Clin Oncol* **26**, 2139-2146, (2008).
137 Jain, N. *et al.* Phase II study of the oral MEK inhibitor selumetinib in advanced acute myelogenous leukemia: a University of Chicago phase II consortium trial. *Clin Cancer Res* **20**, 490-498, (2014).
138 Martelli, A. M. *et al.* Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutical implications for human acute myeloid leukemia. *Leukemia* **20**, 911-928, (2006).

139 Steensma, D. P. *et al.* JAK2 V617F is a rare finding in de novo acute myeloid leukemia, but STAT3 activation is common and remains unexplained. *Leukemia* **20**, 971-978, (2006).
140 Swords, R., Freeman, C. & Giles, F. Targeting the FMS-like tyrosine kinase 3 in acute myeloid leukemia. *Leukemia* **26**, 2176-2185, (2012).
141 Apperley, J. F. Chronic myeloid leukaemia. *Lancet*, (2014).
142 Jain, P., Kantarjian, H. & Cortes, J. Chronic myeloid leukemia: overview of new agents and comparative analysis. *Curr Treat Options Oncol* **14**, 127-143, (2013).
143 Baccarani, M. *et al.* European LeukemiaNet recommendations for the management of chronic myeloid leukemia: 2013. *Blood* **122**, 872-884, (2013).
144 Jabbour, E. & Kantarjian, H. Chronic myeloid leukemia: 2012 update on diagnosis, monitoring, and management. *Am J Hematol* **87**, 1037-1045, (2012).
145 Baccarani, M. *et al.* Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood* **108**, 1809-1820, (2006).
146 Perrotti, D., Jamieson, C., Goldman, J. & Skorski, T. Chronic myeloid leukemia: mechanisms of blastic transformation. *J Clin Invest* **120**, 2254-2264, (2010).
147 Rowley, J. D. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* **243**, 290-293, (1973).
148 Kurzrock, R., Kantarjian, H. M., Druker, B. J. & Talpaz, M. Philadelphia chromosome-positive leukemias: from basic mechanisms to molecular therapeutics. *Ann Intern Med* **138**, 819-830, (2003).
149 Deininger, M. W., Goldman, J. M. & Melo, J. V. The molecular biology of chronic myeloid leukemia. *Blood* **96**, 3343-3356, (2000).
150 Laurent, E., Talpaz, M., Kantarjian, H. & Kurzrock, R. The BCR gene and philadelphia chromosome-positive leukemogenesis. *Cancer Res* **61**, 2343-2355, (2001).
151 Wetzler, M. *et al.* Subcellular localization of Bcr, Abl, and Bcr-Abl proteins in normal and leukemic cells and correlation of expression with myeloid differentiation. *J Clin Invest* **92**, 1925-1939, (1993).
152 Van Etten, R. A. Cycling, stressed-out and nervous: cellular functions of c-Abl. *Trends Cell Biol* **9**, 179-186, (1999).
153 Wang, J. Y. Cellular responses to DNA damage. *Curr Opin Cell Biol* **10**, 240-247, (1998).
154 Laurent, E., Talpaz, M., Wetzler, M. & Kurzrock, R. Cytoplasmic and nuclear localization of the 130 and 160 kDa Bcr proteins. *Leukemia* **14**, 1892-1897, (2000).
155 Pendergast, A. M. *et al.* BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein. *Cell* **75**, 175-185, (1993).
156 Diekmann, D. *et al.* Bcr encodes a GTPase-activating protein for p21rac. *Nature* **351**, 400-402, (1991).
157 O'Hare, T., Deininger, M. W., Eide, C. A., Clackson, T. & Druker, B. J. Targeting the BCR-ABL signaling pathway in therapy-resistant Philadelphia chromosome-positive leukemia. *Clin Cancer Res* **17**, 212-221, (2011).
158 Lugo, T. G., Pendergast, A. M., Muller, A. J. & Witte, O. N. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science* **247**, 1079-1082, (1990).
159 Druker, B. J. *et al.* Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med* **2**, 561-566, (1996).
160 Druker, B. J. *et al.* Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* **344**, 1031-1037, (2001).
161 Kantarjian, H. *et al.* Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N Engl J Med* **346**, 645-652, (2002).
162 O'Brien, S. G. *et al.* Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med* **348**, 994-1004, (2003).
163 Jabbour, E. & Kantarjian, H. Chronic myeloid leukemia: 2014 update on diagnosis, monitoring, and management. *Am J Hematol* **89**, 547-556, (2014).

164 Schindler, T. *et al.* Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. *Science* **289**, 1938-1942, (2000).
165 Savage, D. G. & Antman, K. H. Imatinib mesylate--a new oral targeted therapy. *N Engl J Med* **346**, 683-693, (2002).
166 Capdeville, R., Buchdunger, E., Zimmermann, J. & Matter, A. Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. *Nat Rev Drug Discov* **1**, 493-502, (2002).
167 Druker, B. J. *et al.* Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med* **355**, 2408-2417, (2006).
168 Deininger, M. *et al.* International randomized study of interferon Vs STI571 (IRIS) 8-year follow up: sustained survival and low risk for progression or events in patients with newly diagnosed chronic myeloid leukemia in chronic phase (CML-CP) treated with imatinib. *Blood (ASH Ann Meet Abstr)* **114:Abstract 462**, (2009).
169 Hughes, T. P. *et al.* Long-term prognostic significance of early molecular response to imatinib in newly diagnosed chronic myeloid leukemia: an analysis from the International Randomized Study of Interferon and STI571 (IRIS). *Blood* **116**, 3758-3765, (2010).
170 Marin, D. *et al.* Assessment of BCR-ABL1 transcript levels at 3 months is the only requirement for predicting outcome for patients with chronic myeloid leukemia treated with tyrosine kinase inhibitors. *J Clin Oncol* **30**, 232-238, (2012).
171 le Coutre, P. *et al.* Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. *Blood* **95**, 1758-1766, (2000).
172 White, D. L. *et al.* Chronic phase chronic myeloid leukemia patients with low OCT-1 activity randomized to high-dose imatinib achieve better responses and have lower failure rates than those randomized to standard-dose imatinib. *Haematologica* **97**, 907-914, (2012).
173 Gorre, M. E. *et al.* Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* **293**, 876-880, (2001).
174 Shah, N. P. Loss of response to imatinib: mechanisms and management. *Hematology Am Soc Hematol Educ Program*, 183-187, (2005).
175 Zhou, T. *et al.* Crystal structure of the T315I mutant of Abl kinase. *Chem Biol Drug Des* **70**, 171-181, (2007).
176 Buffa, P. *et al.* BCR-ABL residues interacting with ponatinib are critical to preserve the tumorigenic potential of the oncoprotein. *FASEB J* **28**, 1221-1236, (2014).
177 Zhou, T. *et al.* Structural mechanism of the Pan-BCR-ABL inhibitor ponatinib (AP24534): lessons for overcoming kinase inhibitor resistance. *Chem Biol Drug Des* **77**, 1-11, (2011).
178 Tanaka, R. & Kimura, S. Abl tyrosine kinase inhibitors for overriding Bcr-Abl/T315I: from the second to third generation. *Expert Rev Anticancer Ther* **8**, 1387-1398, (2008).
179 Hochhaus, A. *et al.* Dasatinib induces notable hematologic and cytogenetic responses in chronic-phase chronic myeloid leukemia after failure of imatinib therapy. *Blood* **109**, 2303-2309, (2007).
180 Guilhot, F. *et al.* Dasatinib induces significant hematologic and cytogenetic responses in patients with imatinib-resistant or -intolerant chronic myeloid leukemia in accelerated phase. *Blood* **109**, 4143-4150, (2007).
181 Cortes, J. *et al.* Dasatinib induces complete hematologic and cytogenetic responses in patients with imatinib-resistant or -intolerant chronic myeloid leukemia in blast crisis. *Blood* **109**, 3207-3213, (2007).
182 Shah, N. P. *et al.* Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science* **305**, 399-401, (2004).
183 O'Hare, T. *et al.* In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. *Cancer Res* **65**, 4500-4505, (2005).
184 Tokarski, J. S. *et al.* The structure of Dasatinib (BMS-354825) bound to activated ABL kinase domain elucidates its inhibitory activity against imatinib-resistant ABL mutants. *Cancer Res* **66**, 5790-5797, (2006).
185 Vajpai, N. *et al.* Solution conformations and dynamics of ABL kinase-inhibitor complexes determined by NMR substantiate the different binding modes of imatinib/nilotinib and dasatinib. *J Biol Chem* **283**, 18292-18302, (2008).

186 Weisberg, E. *et al.* Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. *Cancer Cell* **7**, 129-141, (2005).
187 Weisberg, E. *et al.* AMN107 (nilotinib): a novel and selective inhibitor of BCR-ABL. *Br J Cancer* **94**, 1765-1769, (2006).
188 Soverini, S. *et al.* BCR-ABL kinase domain mutation analysis in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors: recommendations from an expert panel on behalf of European LeukemiaNet. *Blood* **118**, 1208-1215, (2011).
189 Khorashad, J. S. *et al.* BCR-ABL1 compound mutations in tyrosine kinase inhibitor-resistant CML: frequency and clonal relationships. *Blood* **121**, 489-498, (2013).
190 Kantarjian, H. *et al.* Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. *N Engl J Med* **354**, 2542-2551, (2006).
191 Kantarjian, H. M. *et al.* Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is effective in patients with Philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase following imatinib resistance and intolerance. *Blood* **110**, 3540-3546, (2007).
192 le Coutre, P. *et al.* Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is active in patients with imatinib-resistant or -intolerant accelerated-phase chronic myelogenous leukemia. *Blood* **111**, 1834-1839, (2008).
193 Levinson, N. M. & Boxer, S. G. Structural and spectroscopic analysis of the kinase inhibitor bosutinib and an isomer of bosutinib binding to the Abl tyrosine kinase domain. *PloS one* **7**, e29828, (2012).
194 Cortes, J. E. *et al.* Safety and efficacy of bosutinib (SKI-606) in chronic phase Philadelphia chromosome-positive chronic myeloid leukemia patients with resistance or intolerance to imatinib. *Blood* **118**, 4567-4576, (2011).
195 Khoury, H. J. *et al.* Bosutinib is active in chronic phase chronic myeloid leukemia after imatinib and dasatinib and/or nilotinib therapy failure. *Blood* **119**, 3403-3412, (2012).
196 Huang, W. S. *et al.* Discovery of 3-[2-(imidazo[1,2-b]pyridazin-3-yl)ethynyl]-4-methyl-N-{4-[(4-methylpiperazin-1-y l)methyl]-3-(trifluoromethyl)phenyl}benzamide (AP24534), a potent, orally active pan-inhibitor of breakpoint cluster region-abelson (BCR-ABL) kinase including the T315I gatekeeper mutant. *J Med Chem* **53**, 4701-4719, (2010).
197 O'Hare, T. *et al.* AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. *Cancer Cell* **16**, 401-412, (2009).
198 Cortes, J. E. *et al.* Ponatinib in refractory Philadelphia chromosome-positive leukemias. *N Engl J Med* **367**, 2075-2088, (2012).
199 Zabriskie, M. S. *et al.* BCR-ABL1 compound mutations combining key kinase domain positions confer clinical resistance to ponatinib in Ph chromosome-positive leukemia. *Cancer Cell* **26**, 428-442, (2014).
200 Cortes, J. E. *et al.* A phase 2 trial of ponatinib in Philadelphia chromosome-positive leukemias. *N Engl J Med* **369**, 1783-1796, (2013).
201 Ariad suspends ponatinib sales. *Cancer Discov* **4**, 6-7, (2014).
202 Small, S. Marketing and commercial distribution of ponatinib suspended following FDA request. *Clin Adv Hematol Oncol* **11**, 808-809, (2013).
203 Miller, G. D., Bruno, B. J. & Lim, C. S. Resistant mutations in CML and Ph(+)ALL - role of ponatinib. *Biologics* **8**, 243-254, (2014).
204 In brief: ponatinib (Inclusig) returns. *Med Lett Drugs Ther* **56**, 8, (2014).
205 Shami, P. J. & Deininger, M. Evolving treatment strategies for patients newly diagnosed with chronic myeloid leukemia: the role of second-generation BCR-ABL inhibitors as first-line therapy. *Leukemia* **26**, 214-224, (2012).
206 Soverini, S. *et al.* Implications of BCR-ABL1 kinase domain-mediated resistance in chronic myeloid leukemia. *Leuk Res* **38**, 10-20, (2014).
207 Shah, N. P. *et al.* Sequential ABL kinase inhibitor therapy selects for compound drug-resistant BCR-ABL mutations with altered oncogenic potency. *J Clin Invest* **117**, 2562-2569, (2007).
208 Zhang, J., Yang, P. L. & Gray, N. S. Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* **9**, 28-39, (2009).
209 Eiring, A. M. & Deininger, M. W. Individualizing kinase-targeted cancer therapy: the paradigm of chronic myeloid leukemia. *Genome Biol* **15**, 461, (2014).

210 Soverini, S. *et al.* Unraveling the complexity of tyrosine kinase inhibitor-resistant populations by ultra-deep sequencing of the BCR-ABL kinase domain. *Blood* **122**, 1634-1648, (2013).
211 Gibbons, D. L. *et al.* Molecular dynamics reveal BCR-ABL1 polymutants as a unique mechanism of resistance to PAN-BCR-ABL1 kinase inhibitor therapy. *Proc Natl Acad Sci U S A* **111**, 3550-3555, (2014).
212 Hu, Y. *et al.* Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. *Nat Genet* **36**, 453-461, (2004).
213 Mullighan, C. G. *et al.* BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature* **453**, 110-114, (2008).
214 Mullighan, C. G. *et al.* Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med* **360**, 470-480, (2009).
215 Martinelli, G. *et al.* IKZF1 (Ikaros) deletions in BCR-ABL1-positive acute lymphoblastic leukemia are associated with short disease-free survival and high rate of cumulative incidence of relapse: a GIMEMA AL WP report. *J Clin Oncol* **27**, 5202-5207, (2009).
216 Iacobucci, I. *et al.* Identification and molecular characterization of recurrent genomic deletions on 7p12 in the IKZF1 gene in a large cohort of BCR-ABL1-positive acute lymphoblastic leukemia patients: on behalf of Gruppo Italiano Malattie Ematologiche dell'Adulto Acute Leukemia Working Party (GIMEMA AL WP). *Blood* **114**, 2159-2167, (2009).
217 Coyaud, E. *et al.* Wide diversity of PAX5 alterations in B-ALL: a Groupe Francophone de Cytogenetique Hematologique study. *Blood* **115**, 3089-3097, (2010).
218 Familiades, J. *et al.* PAX5 mutations occur frequently in adult B-cell progenitor acute lymphoblastic leukemia and PAX5 haploinsufficiency is associated with BCR-ABL1 and TCF3-PBX1 fusion genes: a GRAALL study. *Leukemia* **23**, 1989-1998, (2009).
219 Mullighan, C. G., Williams, R. T., Downing, J. R. & Sherr, C. J. Failure of CDKN2A/B (INK4A/B-ARF)-mediated tumor suppression and resistance to targeted therapy in acute lymphoblastic leukemia induced by BCR-ABL. *Genes Dev* **22**, 1411-1415, (2008).
220 Williams, R. T. & Sherr, C. J. The INK4-ARF (CDKN2A/B) locus in hematopoiesis and BCR-ABL-induced leukemias. *Cold Spring Harb Symp Quant Biol* **73**, 461-467, (2008).
221 Kovacic, B. *et al.* Diverging fates of cells of origin in acute and chronic leukaemia. *EMBO Mol Med* **4**, 283-297, (2012).
222 Lee, H. J., Thompson, J. E., Wang, E. S. & Wetzler, M. Philadelphia chromosome-positive acute lymphoblastic leukemia: current treatment and future perspectives. *Cancer* **117**, 1583-1594, (2011).
223 Fielding, A. K. *et al.* Prospective outcome data on 267 unselected adult patients with Philadelphia chromosome-positive acute lymphoblastic leukemia confirms superiority of allogeneic transplantation over chemotherapy in the pre-imatinib era: results from the International ALL Trial MRC UKALLXII/ECOG2993. *Blood* **113**, 4489-4496, (2009).
224 Liu-Dumlao, T., Kantarjian, H., Thomas, D. A., O'Brien, S. & Ravandi, F. Philadelphia-positive acute lymphoblastic leukemia: current treatment options. *Curr Oncol Rep* **14**, 387-394, (2012).
225 Ottmann, O. G. *et al.* A phase 2 study of imatinib in patients with relapsed or refractory Philadelphia chromosome-positive acute lymphoid leukemias. *Blood* **100**, 1965-1971, (2002).
226 Pfeifer, H. *et al.* Kinase domain mutations of BCR-ABL frequently precede imatinib-based therapy and give rise to relapse in patients with de novo Philadelphia-positive acute lymphoblastic leukemia (Ph+ ALL). *Blood* **110**, 727-734, (2007).
227 Li, S. Src-family kinases in the development and therapy of Philadelphia chromosome-positive chronic myeloid leukemia and acute lymphoblastic leukemia. *Leuk Lymphoma* **49**, 19-26, (2008).
228 Fielding, A. K. How I treat Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood* **116**, 3409-3417, (2010).
229 McDonald, G. O., Stroud, A. N., Brues, A. M. & Cole, W. H. In Vivo and In Vitro Assay for Drug Effect on Cancer Cells. *Annals of surgery* **157**, 785-796, (1963).

230 Larsson, R. & Nygren, P. Laboratory prediction of clinical chemotherapeutic drug resistance: a working model exemplified by acute leukaemia. *Eur J Cancer* **29A**, 1208-1212, (1993).
231 Gustavsson, A. & Olofsson, T. Prediction of response to chemotherapy in acute leukemia by in vitro drug sensitivity testing on leukemic stem cells. *Cancer Res* **44**, 4648-4652, (1984).
232 Larsson, R., Fridborg, H., Kristensen, J., Sundstrom, C. & Nygren, P. In vitro testing of chemotherapeutic drug combinations in acute myelocytic leukaemia using the fluorometric microculture cytotoxicity assay (FMCA). *Br J Cancer* **67**, 969-974, (1993).
233 Pieters, R. *et al.* In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. *Blood* **76**, 2327-2336, (1990).
234 Yamada, S. *et al.* Clinical relevance of in vitro chemoresistance in childhood acute myeloid leukemia. *Leukemia* **15**, 1892-1897, (2001).
235 Tyner, J. W. *et al.* Kinase Pathway Dependence in Primary Human Leukemias Determined by Rapid Inhibitor Screening. *Cancer Res* **73**, 285-296, (2013).
236 Shoemaker, R. H. The NCI60 human tumour cell line anticancer drug screen. *Nat Rev Cancer* **6**, 813-823, (2006).
237 Sharma, S. V., Haber, D. A. & Settleman, J. Cell line-based platforms to evaluate the therapeutic efficacy of candidate anticancer agents. *Nat Rev Cancer* **10**, 241-253, (2010).
238 Barretina, J. *et al.* The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* **483**, 603-607, (2012).
239 Garnett, M. J. *et al.* Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature* **483**, 570-575, (2012).
240 Haibe-Kains, B. *et al.* Inconsistency in large pharmacogenomic studies. *Nature* **504**, 389-393, (2013).
241 Jang, I. S., Neto, E. C., Guinney, J., Friend, S. H. & Margolin, A. A. Systematic assessment of analytical methods for drug sensitivity prediction from cancer cell line data. *Pac Symp Biocomput*, 63-74, (2014).
242 Hatzis, C. *et al.* Enhancing reproducibility in cancer drug screening: how do we move forward? *Cancer Res* **74**, 4016-4023, (2014).
243 Greshock, J. *et al.* Molecular target class is predictive of in vitro response profile. *Cancer Res* **70**, 3677-3686, (2010).
244 Chan, G. K., Kleinheinz, T. L., Peterson, D. & Moffat, J. G. A simple high-content cell cycle assay reveals frequent discrepancies between cell number and ATP and MTS proliferation assays. *PloS one* **8**, e63583, (2013).
245 Fallahi-Sichani, M., Honarnejad, S., Heiser, L. M., Gray, J. W. & Sorger, P. K. Metrics other than potency reveal systematic variation in responses to cancer drugs. *Nat Chem Biol* **9**, 708-714, (2013).
246 Davis, M. I. *et al.* Comprehensive analysis of kinase inhibitor selectivity. *Nat Biotechnol* **29**, 1046-1051, (2011).
247 Shannon, P. *et al.* Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* **13**, 2498-2504, (2003).
248 le Coutre, P. *et al.* In vivo eradication of human BCR/ABL-positive leukemia cells with an ABL kinase inhibitor. *J Natl Cancer Inst* **91**, 163-168, (1999).
249 Koskela, H. L. *et al.* Somatic STAT3 mutations in large granular lymphocytic leukemia. *N Engl J Med* **366**, 1905-1913, (2012).
250 Sulonen, A. M. *et al.* Comparison of solution-based exome capture methods for next generation sequencing. *Genome Biol* **12**, R94, (2011).
251 Koboldt, D. C. *et al.* VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res* **22**, 568-576, (2012).
252 Cingolani, P. *et al.* A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. *Fly* **6**, 80-92, (2012).
253 Kottaridis, P. D. *et al.* The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood* **98**, 1752-1759, (2001).

254 Edgren, H. *et al.* Identification of fusion genes in breast cancer by paired-end RNA-sequencing. *Genome Biol* **12**, R6, (2011).
255 Morrison, J. F. Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors. *Biochim Biophys Acta* **185**, 269-286, (1969).
256 McCoy, A. J. *et al.* Phaser crystallographic software. *J Appl Crystallogr* **40**, 658-674, (2007).
257 Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **66**, 486-501, (2010).
258 Murshudov, G. N., Vagin, A. A. & Dodson, E. J. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* **53**, 240-255, (1997).
259 Adams, P. D. *et al.* PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213-221, (2010).
260 Jing, J. *et al.* Comprehensive predictive biomarker analysis for MEK inhibitor GSK1120212. *Mol Cancer Ther* **11**, 720-729, (2012).
261 Gontarewicz, A. *et al.* Simultaneous targeting of Aurora kinases and Bcr-Abl kinase by the small molecule inhibitor PHA-739358 is effective against imatinib-resistant BCR-ABL mutations including T315I. *Blood* **111**, 4355-4364, (2008).
262 McTigue, M. *et al.* Molecular conformations, interactions, and properties associated with drug efficiency and clinical performance among VEGFR TK inhibitors. *Proc Natl Acad Sci U S A* **109**, 18281-18289, (2012).
263 Rini, B. I. *et al.* Comparative effectiveness of axitinib versus sorafenib in advanced renal cell carcinoma (AXIS): a randomised phase 3 trial. *Lancet* **378**, 1931-1939, (2011).
264 Wang, G. G., Cai, L., Pasillas, M. P. & Kamps, M. P. NUP98-NSD1 links H3K36 methylation to Hox-A gene activation and leukaemogenesis. *Nat Cell Biol* **9**, 804-812, (2007).
265 Cerveira, N. *et al.* Frequency of NUP98-NSD1 fusion transcript in childhood acute myeloid leukaemia. *Leukemia* **17**, 2244-2247, (2003).
266 Hollink, I. H. *et al.* NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern. *Blood* **118**, 3645-3656, (2011).
267 Fasan, A. *et al.* A rare but specific subset of adult AML patients can be defined by the cytogenetically cryptic NUP98-NSD1 fusion gene. *Leukemia* **27**, 245-248, (2013).
268 Dowling, R. J. *et al.* mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. *Science* **328**, 1172-1176, (2010).
269 Lannon, C. L. *et al.* A highly conserved NTRK3 C-terminal sequence in the ETV6-NTRK3 oncoprotein binds the phosphotyrosine binding domain of insulin receptor substrate-1: an essential interaction for transformation. *J Biol Chem* **279**, 6225-6234, (2004).
270 Morrison, K. B., Tognon, C. E., Garnett, M. J., Deal, C. & Sorensen, P. H. ETV6-NTRK3 transformation requires insulin-like growth factor 1 receptor signaling and is associated with constitutive IRS-1 tyrosine phosphorylation. *Oncogene* **21**, 5684-5695, (2002).
271 Tognon, C. E. *et al.* A tripartite complex composed of ETV6-NTRK3, IRS1 and IGF1R is required for ETV6-NTRK3-mediated membrane localization and transformation. *Oncogene* **31**, 1334-1340, (2012).
272 Tamburini, J. *et al.* Mammalian target of rapamycin (mTOR) inhibition activates phosphatidylinositol 3-kinase/Akt by up-regulating insulin-like growth factor-1 receptor signaling in acute myeloid leukemia: rationale for therapeutic inhibition of both pathways. *Blood* **111**, 379-382, (2008).
273 Solowiej, J. *et al.* Characterizing the effects of the juxtamembrane domain on vascular endothelial growth factor receptor-2 enzymatic activity, autophosphorylation, and inhibition by axitinib. *Biochemistry* **48**, 7019-7031, (2009).
274 Azam, M., Seeliger, M. A., Gray, N. S., Kuriyan, J. & Daley, G. Q. Activation of tyrosine kinases by mutation of the gatekeeper threonine. *Nat Struct Mol Biol* **15**, 1109-1118, (2008).

275 Dixit, A. & Verkhivker, G. M. Hierarchical modeling of activation mechanisms in the ABL and EGFR kinase domains: thermodynamic and mechanistic catalysts of kinase activation by cancer mutations. *PLoS Comput Biol* **5**, e1000487, (2009).
276 Basu, A. *et al.* An interactive resource to identify cancer genetic and lineage dependencies targeted by small molecules. *Cell* **154**, 1151-1161, (2013).
277 Heiser, L. M. *et al.* Subtype and pathway specific responses to anticancer compounds in breast cancer. *Proc Natl Acad Sci U S A* **109**, 2724-2729, (2012).
278 Martins, M. M. *et al.* Linking tumor mutations to drug responses via a quantitative chemical-genetic interaction map. *Cancer Discov* **5**, 154-167, (2015).
279 Scholl, C., Gilliland, D. G. & Frohling, S. Deregulation of signaling pathways in acute myeloid leukemia. *Semin Oncol* **35**, 336-345, (2008).
280 Stirewalt, D. L. *et al.* FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. *Blood* **97**, 3589-3595, (2001).
281 Druker, B. J. Perspectives on the development of imatinib and the future of cancer research. *Nat Med* **15**, 1149-1152, (2009).
282 Kandoth, C. *et al.* Mutational landscape and significance across 12 major cancer types. *Nature* **502**, 333-339, (2013).
283 Hoshino, R. *et al.* Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* **18**, 813-822, (1999).
284 Towatari, M. *et al.* Constitutive activation of mitogen-activated protein kinase pathway in acute leukemia cells. *Leukemia* **11**, 479-484, (1997).
285 Lee, J. T., Jr. & McCubrey, J. A. The Raf/MEK/ERK signal transduction cascade as a target for chemotherapeutic intervention in leukemia. *Leukemia* **16**, 486-507, (2002).
286 Ricciardi, M. R. *et al.* Therapeutic potential of MEK inhibition in acute myelogenous leukemia: rationale for "vertical" and "lateral" combination strategies. *J Mol Med (Berl)*, (2012).
287 Milella, M. *et al.* Beyond single pathway inhibition: MEK inhibitors as a platform for the development of pharmacological combinations with synergistic anti-leukemic effects. *Curr Pharm Des* **11**, 2779-2795, (2005).
288 Lauchle, J. O. *et al.* Response and resistance to MEK inhibition in leukaemias initiated by hyperactive Ras. *Nature* **461**, 411-414, (2009).
289 McKay, M. M., Ritt, D. A. & Morrison, D. K. Signaling dynamics of the KSR1 scaffold complex. *Proc Natl Acad Sci U S A* **106**, 11022-11027, (2009).
290 Dry, J. R. *et al.* Transcriptional pathway signatures predict MEK addiction and response to selumetinib (AZD6244). *Cancer Res* **70**, 2264-2273, (2010).
291 Zhao, S. *et al.* Inhibition of phosphatidylinositol 3-kinase dephosphorylates BAD and promotes apoptosis in myeloid leukemias. *Leukemia* **18**, 267-275, (2004).
292 Staber, P. B. *et al.* Common alterations in gene expression and increased proliferation in recurrent acute myeloid leukemia. *Oncogene* **23**, 894-904, (2004).
293 Zeng, Z. *et al.* Rapamycin derivatives reduce mTORC2 signaling and inhibit AKT activation in AML. *Blood* **109**, 3509-3512, (2007).
294 Kornblau, S. M. *et al.* Simultaneous activation of multiple signal transduction pathways confers poor prognosis in acute myelogenous leukemia. *Blood* **108**, 2358-2365, (2006).
295 Nyakern, M. *et al.* Frequent elevation of Akt kinase phosphorylation in blood marrow and peripheral blood mononuclear cells from high-risk myelodysplastic syndrome patients. *Leukemia* **20**, 230-238, (2006).
296 Recher, C. *et al.* Antileukemic activity of rapamycin in acute myeloid leukemia. *Blood* **105**, 2527-2534, (2005).
297 Yee, K. W. *et al.* Phase I/II study of the mammalian target of rapamycin inhibitor everolimus (RAD001) in patients with relapsed or refractory hematologic malignancies. *Clin Cancer Res* **12**, 5165-5173, (2006).
298 Perl, A. E. *et al.* A phase I study of the mammalian target of rapamycin inhibitor sirolimus and MEC chemotherapy in relapsed and refractory acute myelogenous leukemia. *Clin Cancer Res* **15**, 6732-6739, (2009).
299 Kropf, P. L., Wang, L., Zang, Y., Redner, R. L. & Johnson, D. E. Dasatinib promotes ATRA-induced differentiation of AML cells. *Leukemia* **24**, 663-665, (2010).
300 Chevalier, N. *et al.* Robust in vivo differentiation of t(8;21)-positive acute myeloid leukemia blasts to neutrophilic granulocytes induced by treatment with dasatinib. *Leukemia* **24**, 1779-1781, (2010).

301 Guerrouahen, B. S. *et al.* Dasatinib inhibits the growth of molecularly heterogeneous myeloid leukemias. *Clin Cancer Res* **16**, 1149-1158, (2010).
302 Lainey, E. *et al.* Tyrosine kinase inhibitors for the treatment of acute myeloid leukemia: delineation of anti-leukemic mechanisms of action. *Biochem Pharmacol* **82**, 1457-1466, (2011).
303 Knezevich, S. R., McFadden, D. E., Tao, W., Lim, J. F. & Sorensen, P. H. A novel ETV6-NTRK3 gene fusion in congenital fibrosarcoma. *Nat Genet* **18**, 184-187, (1998).
304 Liu, Q. *et al.* Signal transduction and transforming properties of the TEL-TRKC fusions associated with t(12;15)(p13;q25) in congenital fibrosarcoma and acute myelogenous leukemia. *EMBO J* **19**, 1827-1838, (2000).
305 Tognon, C. *et al.* Expression of the ETV6-NTRK3 gene fusion as a primary event in human secretory breast carcinoma. *Cancer Cell* **2**, 367-376, (2002).
306 Mayer, K., Gielen, G. H., Willinek, W., Muller, M. C. & Wolf, D. Fatal progressive cerebral ischemia in CML under third-line treatment with ponatinib. *Leukemia* **28**, 976-977, (2014).
307 Chan, W. W. *et al.* Conformational control inhibition of the BCR-ABL1 tyrosine kinase, including the gatekeeper T315I mutant, by the switch-control inhibitor DCC-2036. *Cancer Cell* **19**, 556-568, (2011).
308 Gross-Goupil, M., Francois, L., Quivy, A. & Ravaud, A. Axitinib: a review of its safety and efficacy in the treatment of adults with advanced renal cell carcinoma. *Clin Med Insights Oncol* **7**, 269-277, (2013).
309 Bracarda, S. *et al.* Axitinib safety in metastatic renal cell carcinoma: suggestions for daily clinical practice based on case studies. *Expert Opin Drug Saf* **13**, 497-510, (2014).
310 Verzoni, E. *et al.* Targeted treatments in advanced renal cell carcinoma: focus on axitinib. *Pharmgenomics Pers Med* **7**, 107-116, (2014).
311 Josephs, D. H., Fisher, D. S., Spicer, J. & Flanagan, R. J. Clinical pharmacokinetics of tyrosine kinase inhibitors: implications for therapeutic drug monitoring. *Ther Drug Monit* **35**, 562-587, (2013).
312 Chen, Y. *et al.* Clinical pharmacology of axitinib. *Clin pharmacokinet* **52**, 713-725, (2013).
313 Shah, N. P. *et al.* Intermittent target inhibition with dasatinib 100 mg once daily preserves efficacy and improves tolerability in imatinib-resistant and -intolerant chronic-phase chronic myeloid leukemia. *J Clin Oncol* **26**, 3204-3212, (2008).
314 O'Hare, T. *et al.* Threshold levels of ABL tyrosine kinase inhibitors retained in chronic myeloid leukemia cells determine their commitment to apoptosis. *Cancer Res* **73**, 3356-3370, (2013).
315 Schmidinger, M. Understanding and managing toxicities of vascular endothelial growth factor (VEGF) inhibitors. *EJC Supplements* **11**, 172-191, (2013).